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Virus of Avian Erythromyeloblastic Leukosis. I. Relation of Specific Plasma Particles to the Dephosphorylation of Adenosine Triphosphate [1, 2]

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The viral etiology of avian erythromyeloblastic leukosis (*1*) has long been known (*2*), and the presence of the infectious agent in the blood plasma has been confirmed repeatedly (*3, 4*). Recently a particulate component was found (*5, 6*), occurring in variable numbers in the plasmas of diseased chicks. A third attribute of the plasmas, discovered still more recently (*7*), is a strong capacity to dephosphorylate adenosine triphosphate.

With the demonstration of the particulate component in association with the specific infectious properties of the plasmas, the particles and the virus were regarded as identical. Continued study of the enzymatic activity produced increasing evidence of an intimate relation, likewise, between the particles and the enzyme. These findings suggested that the plasma particles might constitute the virus and that both infectious capacity and enzyme activity are properties of the virus particle. Inasmuch as viruses have been notoriously lacking in demonstrable enzyme systems, it became essential to investigate in detail the characters of the interrelations between these three plasma attributes—infectious capacity, particle content, and enzymatic activity. The principal purpose of the present report is a description of the relationship between the particles, counted by electron microscopy, and the dephosphorylating activity, as they occur in the plasmas of chicks infected experimentally with the virus. Studies on electrophoresis and sedimentation of the particles and the enzyme and of the infectious entity will be described in separate reports (*8, 9*).

Materials and Methods

The origin and character of the disease under study have been given in earlier publications (*4, 10*). The chicks were a special strain of White

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[2] This work was supported by a research grant to Duke University from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service; by a grant from the American Cancer Society, on recommendation of the Committee on Growth; by the Dorothy Beard Research Fund; it was also sponsored by the Commission on Influenza, Armed Forces Epidemiological Board, and supported, in part, by the Office of The Surgeon General, Department of the Army.

[3] Scholar in Cancer Research of the American Cancer Society, Inc.

Leghorns, line 15, developed (*11*) at the Regional Poultry Research Laboratory at East Lansing, Michigan. These animals were chosen, as will be described later (*12,13*), because of an exceedingly high susceptibility to the virus of erythromyeloblastic leukosis.

Chick donors furnishing the plasmas were taken from various current experiments involving titration of the virus, in which previous passage plasma in various dilutions was injected in 0.1-ml. volumes in 3-day-old chicks. Occurrence of the disease was determined by daily blood smears (*4*), and prospective donors were chosen first on the basis of high blood content of primitive cells and selected finally for the experiments by the results of the micro screening test based on the activity of the dephosphorylating enzyme (*14*). The birds were bled by heart puncture or incision through the saggital sinus. Blood was collected in graduated, pointed centrifuge tubes with heparin (Liquaemin sodium, Organon, Inc., Orange, N. J.) in amounts of 10 mg. per 10 ml. of blood. The cells were promptly sedimented by centrifugation for 15 minutes at 1,029 $\times$ g, and an approximate estimate of the plasma–cell volume relationship was obtained. The plasma was pipetted off and spun again in celluloid tubes at 3,715 $\times$ g for 15 minutes. The plasma, removed carefully to avoid residual sedimented cells, was diluted 1:1 or 1:2 with Ca-free Ringer's solution. A small amount of celite #512 was shaken into the fluid which was then passed with suction through a 2- or 3-mm. mat of the celite supported on coarse filter paper in a Hirsch funnel having 12 mm. of perforated area. This was followed by filtration, with suction, through small Selas filters (#MFI–11, porosity 02, Selas Corporation of America, Scientific Equipment Division, Philadelphia, Pa.). During all the manipulations, the tubes containing the materials were either handled in the refrigerator or kept in an ice bath. Enzyme studies and particle counts were made within a few hours after the plasma was filtered or, in a few instances, on the filtered plasma held overnight in the refrigerator.

Dephosphorylating activity was determined essentially by the method of Green and Mommaerts (*15*). The reaction was studied at 30° C. in volumes of 20 ml. comprised, in one group of experiments, of 0.2 ml. of 1.0 *M* $CaCl_2$ solution and the necessary volume of Ca-free Ringer's solution to make the required total with 0.05 to 0.2 ml. of plasma and 1.0 ml. of adenosine triphosphate solution. In the other experiments, the $CaCl_2$ was omitted, and its place was taken by the equal additional volume of Ca-free Ringer's solution. The plasma, in the 19.0-ml. volume, was incubated for 3 minutes at 30° C., and the adenosine triphosphate solution, at *p*H 9 and containing 4 mg. of the disodium salt per ml., was quickly added. The rate of dephosphorylation was followed titrimetrically with the glass electrode and Beckman *p*H meter, employing *p*H 8.0 as the index point, on addition of a total of 0.3 ml. of 0.01 *N* KOH in successive 0.05-ml. aliquots. These data were transformed to an expression of the rate of the liberation of phosphorus per unit volume of plasma, giving results found to be identical with those obtained in the direct determination of phosphorus with the method of Berenblum and Chain (*16*).

Plasma particles were counted by electron microscopy after sedimentation on agar (*6*, *17*, *18*), with minor modifications of the procedure adapted to the problem at hand. After filtration, the plasma was diluted with 0.85 percent NaCl solution containing 0.4 percent formaldehyde in the range of 1:50 to 1:500 (total plasma dilution 1:100 to 1:1,000), depending on the number of particles. Aggregation of the particles occurred occasionally under these conditions, but redispersion could be effected by brief exposure of the preparation to sonic vibration at 9 kilocycles.[4] Agar was prepared in 10-ml. lots at 3 percent concentration in distilled water. To this volume of melted agar there were added 2 ml. of 1 *M* phosphate buffer of *p*H 7.0 and 8 ml. of the 0.85 percent NaCl-formaldehyde solution, and the mixture (now 1.5 percent agar) was poured into a 4-inch petri dish. The plate was allowed to stand at room temperature overnight. Blocks were cut for the ultracentrifuge cells, and the particles were sedimented (*18*) at 16,400 × g for 30 minutes at room temperature. The particles on the agar surface, after the block was removed from the cell, were fixed with the vapor from 2 percent osmic acid for 15 minutes. Collodion in 1 percent concentration in amyl acetate was employed for forming the film for the pseudoreplica. The procedures for floating the film off the agar and shadowcasting the particles were those previously used (*18*). At least 5 exposures, at 3,500 × magnification, were made of each preparation in the electron microscope, and the particle numbers cited are the averages of the counts of all the particles.

Results

The origin, various features and properties of the plasmas, and the results obtained in the studies are given in tables 1 and 2. Table 1 includes the plasmas from chicks varying from 19 to 31 days in age at the time the blood was drawn. The summary of the analogous characters of plasmas from older chicks, varying from 34 to 80 days of age, appears in table 2. This separation of the donor chicks at the 31-day age level is an arbitrary one, the reasons for which will be explained with the presentation of the data. All of the experiments were made in the period of 7 weeks between 12/22/52 and 2/6/53.

Several differences are seen between the younger and the older chicks. In the former, the incubation period was short, varying from 12 to 20 days with an average of 15.9 days. In contrast, the older chicks, which had been injected initially like the younger ones at 3 days of age, became ill after much longer intervals—22 to 64 days with an average of 42.8 days. There were large differences between the younger and the older chicks in the duration of the disease before bleeding as shown by the averages of 5.8 and 15.5 days, and in the chronological ages of the two groups at the time of bleeding, namely, an average of 24.7 days compared with 61.3 days for the older chicks. The birds having the shortest incubation periods usually died quickly, while the older chicks, which were more highly resistant or received less virus, were much more able to survive

[4] The use of formaldehyde and sonic vibration has been discontinued in more recent work.

TABLE 1.—*The characters of and the results obtained with the plasmas from donor chicks of 19 to 31 days of age*

Donor No.	Donor inoculum*	Incubation period	Period positive	Donor age	Micro test	Cell volume	Plasma volume	Particle count	Enzyme activity	
									with Ca	without Ca
		(*days*)	(*days*)	(*days*)	(*min.*)	(*ml.*)	(*ml.*)	(× 10^9)	(γ *P*)	(γ *P*)
S 64	P 10^{-1}	16	4	23	10	1.2	1.2	240	92	28
S 61	P 10^{-1}	16	4	23	.....	1.9	1.9	211	158	63
S 50	P 10^{-1}	16	3	22	5	1.0	1.0	51	.....	24
S 30	P 10^{0}	16	3	22	10	1.3	1.1	85	.....	19
R 355	P $10^{-1.4}$	19	9	31	5	1.4	3.1	518	.....	132
S 939	P 10^{-1}	12	4	19	9	1.9	2.9	233	194	.....
S 700	P 10^{-1}	15	6	24	.....	2.0	3.0	217	143	.....
T 50	P 10^{-2}	16	7	26	5	2.1	2.6	832	661	.....
T 77	P 10^{-2}	17	6	26	5	1.7	2.3	625	505	.....
S 842	P 10^{-1}	19	4	26	10	2.2	2.1	352	232	.....
R 500	P 10^{0}	17	7	27	5	3.4	4.1	125	102	20
R 504	P 10^{0}	17	8	28	10	2.2	1.1	526	446	121
R 510	P 10^{-1}	17	7	27	5	2.1	3.2	319	179	74
R 526	P 10^{-1}	20	4	27	5	3.0	2.7	454	424	127
R 534	P 10^{-1}	19	5	27	5	3.6	2.8	533	537	162
R 536	P 10^{-1}	14	11	28	10	4.4	2.8	525	344	132
R 524	P 10^{-1}	15	9	27	5	3.4	3.3	665	400	165
R 557	P 10^{-1}	17	8	28	10	2.2	3.2	167	97	29
R 592	P 10^{-2}	17	8	28	10	4.1	2.2	597	391	123
R 624	IS_1	17	7	27	5	3.5	2.6	207	90	30
R 640	IS_1	17	6	26	10	4.0	2.2	184	144	60
R 655	IS_1	15	9	27	5	3.2	1.8	647	554	135
R 657	IS_1	17	8	28	10	3.1	3.5	245	148	56
R 675	IS_1	18	7	28	10	2.1	2.3	201	91	24
R 793	P 10^{0}	13	5	21	5	1.0	1.3	217	195	59
R 797	P 10^{0}	14	4	21	5	1.4	1.4	457	337	.....
R 801	P 10^{0}	13	6	22	10	1.0	1.8	169	164	.....
R 851	P 10^{-1}	14	4	21	5	0.6	1.0	290	226	.....
R 977	IS_3	13	5	21	5	1.7	2.1	859	731	.....
S 244	IS_2	16	4	23	10	1.4	2.1	244	124	77
S 212	IS_2	16	4	23	15	1.1	1.9	160	59	30
S 190	IS_2	16	4	23	10	2.5	2.5	253	109	34
S 187	IS_2	13	7	23	10	1.8	2.8	302	118	46
S 177	IS_1	16	4	23	10	1.6	2.4	160	153	34
S 76	P 10^{-1}	16	3	22	10	2.1	1.6	180	.....	43
S 66	P 10^{-1}	13	5	21	10	1.6	2.3	171	.....	46

*P 10^{-1} signifies that donor had received 0.1 ml. of plasma diluted 10^{-1}; IS indicates that the plasma inoculum contained antileukosis virus rabbit serum.

the grave anemia and the unknown factors associated with the disease. Large numbers of the youngest birds died too quickly to be of use in these experiments. Those in table 1 were about the youngest available for routine study.

The tables also show the results of the micro screening test, which may be useful for various correlations with the findings reported earlier (*14*).

The plasma yields, approximately the volumes indicated by the hematocrit, varied with the age of the chick, being, in some instances, scarcely more than 1.0 ml. The volume of diluted plasma passed through the Selas filter was usually 2 to 4 ml. Filtration was most often relatively rapid, requiring 6 to 30 minutes. Some of the plasmas, however, were highly viscous, due apparently to the presence of fibrin strands which could be seen in electron micrographs as illustrated in figure 1. In these

TABLE 2.—*The characters of and the results obtained with the plasmas from donor chicks of 3½ to 80 days of age*

Donor No.	Donor inoculum*	Incubation period	Period positive	Donor age	Micro test	Cell volume	Plasma volume	Particle count	Enzyme activity	
									with Ca	without Ca
		(*days*)	(*days*)	(*days*)	(*min.*)	(*ml.*)	(*ml.*)	(× 10^9)	(γ *P*)	(γ *P*)
R 299	P 10^{-2}	33	20	56	5	6. 5	5. 5	501	.....	65
R 419	P 10^{-3}	27	20	50	5	7. 7	1. 7	178	.....	68
R 873	P 10^{0}	26	13	42	5	1. 1	0. 9	422	.....	41
R 960	IS_2	29	10	42	5	3. 6	5. 2	404	.....	28
N 692	P 10^{0}	64	10	77	15	8. 5	7. 5	51	.....	16
N 734	P 10^{0}	64	10	77	15	4. 3	2. 9	10	54	0
N 746	P 10^{0}	60	11	74	10	2. 2	2. 6	47	57	0
N 814	P 10^{-1}	54	17	74	15	1. 4	1. 5	46	121	0
N 820	P 10^{-1}	60	13	76	15	9. 9	8. 5	330	.....	32
N 869	P 10^{-2}	54	20	77	10	6. 5	6. 5	5	132	0
O 123	P 10^{-2}	43	24	70	15	5. 7	6. 0	90	.....	11
O 310	P 10^{-2}	35	24	61	15	10. 2	6. 7	191	.....	13
O 139	P 10^{-2}	57	11	71	15	18. 1	12. 2	105	98	24
O 326	P 10^{-3}	55	6	64	10	12. 5	17. 5	139	25	0
O 341	P 10^{-3}	43	18	64	10	6. 0	4. 5	210	134	29
O 354	P 10^{-3}	43	34	80	3	6. 9	2. 5	792	.....	716
S 365	P 10^{-2}	38	4	45	5	10. 1	3. 9	217	.....	124
S 757	P 10^{-2}	22	9	34	11	3. 6	2. 8	275	.....	161
R 906	IS_1	39	14	56	.....	3. 2†	1. 8†	332	.....	131
S 379	IS_1	25	8	36	.....	6. 2	1. 8	320	.....	289
R 420	P 10^{-3}	27	28	58	5	12. 2	13. 8	347	312	91
O 406	IS_2	43	18	64	10	32. 8	7. 2	421	264	77

*P 10^{-1} signifies that donor had received 0.1 ml. of plasma diluted 10^{-1}; IS indicates that the plasma inoculum contained antileukosis virus rabbit serum.

†Hematocrit observed in the micro screening test as described elsewhere (*14*).

instances, the celite gathered into rapidly sedimenting agglomerates, and the liquid passed with difficulty through the celite mat, requiring repeated efforts with several mats. These plasmas were likewise difficult to get through the Selas filter, and often several filters were used for a single specimen. The extent of the loss of enzyme activity in the filtration process was not studied in detail; the results of the few experiments given in table 3 indicated that significant loss was infrequent in the combined celite and Selas filtration, and this occurred with the plasmas most difficult to filter. It is seen in table 1 that the concentration of particles in some of the plasmas was exceedingly high, and highly active plasmas were readily recognized visually by cloudiness or opacity of the preparation due to the particles.

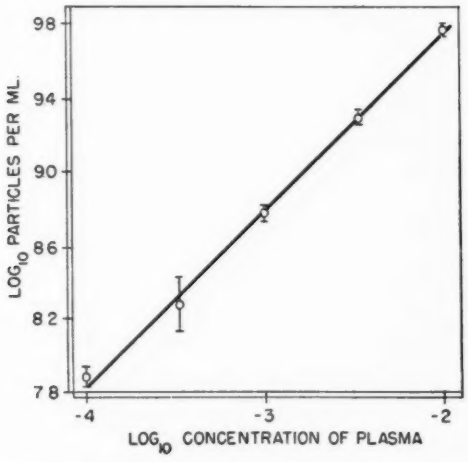
The conditions for the study of dephosphorylation of adenosine triphosphate were established in experiments which will be reported in detail in a separate publication. For the present purpose there are cited the major factors influencing the reaction. A principal one of these is the marked effect of calcium in increasing the activity of the enzyme. A similar though lesser effect was seen with magnesium. The amount of calcium present in the plasma in the larger volumes employed, 0.2 ml., was sufficient to influence the reaction. Consequently, it was regarded as necessary to study the reaction with some of the plasmas in the pres-

TABLE 3.—*Enzyme activity of plasmas before and after filtration through celite and Selas filters* (γ *P liberated per minute per ml. plasma*)

Plasma	Enzyme activity	
	Before filtration (γ *P*)	After filtration (γ *P*)
R 299	63	65
R 355	132	138
R 960	31	28
R 906	221	131
T 50	661	661
S 842	235	232
R 430	452	370
T 77	529	505
T 62	196	162
W 188	343	223
W 19	573	456

ence of calcium added to optimum concentration, 0.01 *M*, as well as in a reaction medium without added calcium. While the optimum *p*H for the activity was found to be in the region of 6.8 to 7.0, the index point of *p*H 8 was chosen on the basis of theoretical considerations (*15*). In the enzymatic dephosphorylation of adenosine triphosphate by myosin, the equivalence ratio of acid to PO_4 is unity, whereas in the region of *p*H 7.0 the ratio is less than one. Variation in ionic concentration by progressive dilutions of the basic Ca-free Ringer's solution as much as 100-fold, or by addition of NaCl to concentrations 100 times the physiological level, did not influence the rate of reaction. The stability of the enzyme in the plasmas was variable, the activity diminishing significantly in some within a few hours and in others remaining stable for several days. Possible loss of activity was not determined with the plasmas in the present work, but studies were made in all instances as soon as practical.

The procedure for counting the plasma particles has been greatly improved during the past two years in studies for the determination of optimum conditions for counting the particles of this virus. The gravitational field and the time of sedimentation employed were slightly above the levels required for deposit of the maximum number of particles on the agar surface. Little influence was shown by differences in the *p*H of the buffered agar in the region of 5 to 7. It would be very difficult to establish the accuracy of the method; the reproducibility of the results under a given set of conditions, however, may be judged from the counts made by centrifuging various dilutions of a given plasma. The points of text-figure 1 are the average of 5 exposures at each dilution. There are indicated, also, the limits of variation among the individual exposures at each dilution. In this and other experiments it was seen that the variation is small and that dilution of such a plasma of high count did not influence the result.

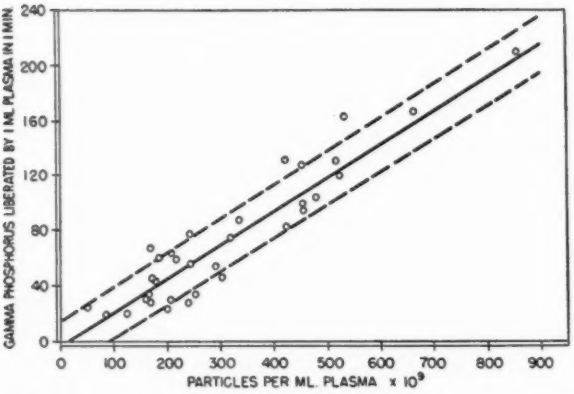


TEXT-FIGURE 1.—Enumeration of plasma particles sedimented from different dilutions of the same plasma.

In contrast with this lack of effect of dilution of a good plasma are the difficulties experienced in the counting with plasmas of low particle content. Here, the dilution factor for counting is low, and extraneous material sedimented with the specific particles results in uncertainty. This was not an influence with the greater dilutions possible with the plasmas of higher particle content. The potentialities for accurate counting are clearly illustrated in the electron micrographs of figures 2 and 3. The former shows a micrograph of a plasma of low particle content diluted 1:100 with which an accurate count is clearly not possible. The latter micrograph is one of a plasma which could be diluted 1:2,000 and with which there is little problem in counting.

The relationships of dephosphorylating activity to the particle number per unit volume of plasma for the younger group of chicks are given in tables 1 and 2 and shown graphically, in the absence of added calcium, in text-figure 2, and in the presence of calcium added to optimal concentration, in text-figure 3. It is seen that the relationship is very close, especially in the range of the higher levels of activity and particle number. The natural limits of the dephosphorylating activity restricted the studies, under the conditions employed, to those specimens containing about 10^9 and more particles per ml. Not all of the plasmas of text-figure 2 were the same as those of text-figure 3; the specimens of lowest particle content could not be studied adequately in the volumes employed without added calcium except by increase in the volume of plasma and consequent introduction of unknown amounts of blood calcium.

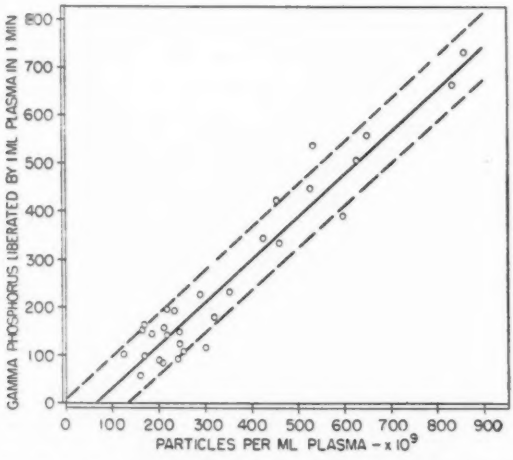
An indication that the relationship is linear is suggested by the close fit of the individual points to the line drawn by the method of least squares. The areas enclosed by the discontinuous lines in both text-figures represent the respective fiduciary bands indicating the limits of the expected varia-



TEXT-FIGURE 2.—Relationship of enzyme activity in the absence of added calcium to particle content of plasmas from chicks of 19 to 31 days of age.

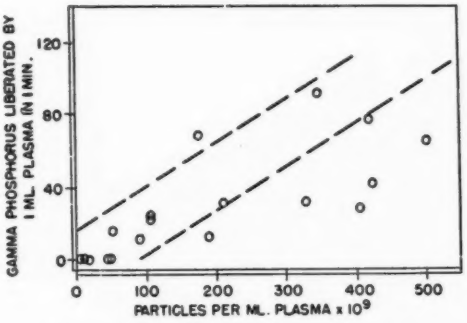
tion at a probability of 0.66 in the relationship in a single determination of enzymatic activity and particle count. In 2 of 3 such cases the variation expected in the absence of added calcium is ± 20 gamma phosphorus for a given particle count or $\pm 80 \times 10^9$ particles for a given enzyme activity. The variations for studies in the presence of added calcium are ± 70 gamma phosphorus or $\pm 79 \times 10^9$ particles. Since the absolute variation is uniform over the whole range, the percentage variation becomes progressively less with the more active plasmas.

Enzyme activities and particle counts of the plasmas from the older chicks (table 2) are compared in text-figures 4 (no calcium added) and 5 (calcium added). In text-figures 4 and 5, there are reproduced the same

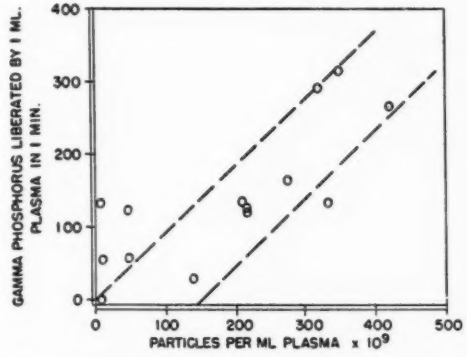


TEXT-FIGURE 3.—Relationship of enzyme activity in the presence of added calcium to particle content of plasmas from chicks of 19 to 31 days of age.

fiduciary bands as those of text-figures 2 and 3, respectively. Here it is evident that the spread of the individual points is greater than those obtained with the plasmas from the younger chicks. When enzyme activity was measured without the addition of calcium, the results in at least 4 instances were well outside the range of variation seen with the specimens from the younger donors. This variation was in the direction of lower activity in relation to particle count. The reverse was true when calcium was added, in which instance the enzyme activity seemed high.



TEXT-FIGURE 4.—Relationship of enzyme activity in the absence of added calcium to particle content of plasmas from chicks of 34 to 80 days of age.



TEXT-FIGURE 5.—Relationship of enzyme activity in the presence of added calcium to particle content of plasmas from chicks of 34 to 80 days of age.

Discussion

The results demonstrate a close and, apparently, linear relationship between the enzyme activity to dephosphorylate adenosine triphosphate and the particle content of plasmas from young chicks with erythromyeloblastic leukosis. Of special significance is the fact that these two qualities, enzyme activity and particle number, are proportional in their *occurrence under natural conditions* in the development of the disease in the chicks. This finding provides one facet of quantitative evidence pointing

to the plasma particles specific to the disease as the source of the enzyme. It is notable that the plasmas received no special treatment other than the filtration process, which was regarded to be essential in the elimination of cellular material as a source of the enzyme.

Greater variation was seen with the group of older chicks. Enzyme activity in the absence of calcium (text-figure 4) was definitely less than expected in some instances. In the presence of calcium there were some variations in the opposite direction. The separation of the results with the plasmas of the older chicks from those of the younger was based not only on the present work but on other experiments (*17*) indicating that the properties of the particles may be different in older chicks from those of the particles from young ones. On the basis of general experience as well as the present results it would appear well to recognize the possibility that significant differences may exist. This is especially true in the face of the present variation despite the deliberate selection with the micro screening test (*14*) of older birds of high plasma activity.

It is not possible to decide on the significance of the observed degree of correlation. Variations were seen both in the absence of added calcium and when calcium was present in optimal concentration. In the region of low particle content, where the results of counting may be most erratic (fig. 2), the ratio of particles to enzyme activity might vary to extremes of approximately 100 percent. In contrast, it seems significant that the variation declined to the order of only 10 to 20 percent in the results with plasmas of high particle content. No standard of reference is available for judging how close the correlation ought to be if the enzyme activity were an intrinsic property of the particle. One possible basis for comparison is the experience in the study of the dephosphorylation of adenosine triphosphate by purified myosin, which is regarded as a source of the enzyme. In this case, under optimum conditions, variation in the ratio of activity to myosin is approximately ± 30 percent (*19*). The average variations in the present work were certainly within this magnitude.

A consideration of the variations must include not only those inherent in the properties of the materials studied but those resident in the methods of study. The determination of enzyme activity under given conditions seems of considerable precision (see table 3). On the other hand, the factors possibly contributing to variation from one plasma to another are not known. A definite indication of the present lack of complete control of the reaction or the knowledge of the factors influencing it is the variation in the ratio of activity in the presence of the optimum concentration of calcium to that where no calcium was added. From calculations with the values of table 1, the variation at the extremes of activity is 5.1 to 1.6 though most of the ratios lie in the range of 2.4 to 3.5. It seems probable that this was not due to technical errors, but to actual differences in the enzyme activities of different populations of particles or in some other factors that might influence the activity. It is notable in this respect that the calcium activation of the enzyme associated with myosin is likewise variable (*19*).

It has already been recognized that the technique of particle counting can be the source of much variation. While every effort has been made to control the procedure, it is increasingly evident that the absolute count is influenced by many small factors such as, for example, the concentration of agar, different batches of agar, how long the agar has been prepared, how long the plate stands before use, and many others not yet detected. The lack of a permanent standard or control has prevented the establishment of optimum, reproducible conditions. It has been the experience in this laboratory that while the precision of the method may vary with change in conditions, causing errors in absolute values, relative values of close proportionality are readily obtainable when a given set of conditions is rigidly maintained, as in the present work.

Despite the possible contributions of technical errors to the observed variations, it still remains likely that part of the variation may be founded on a biological basis. Even if it were known that the enzyme activity is a property of the particle, it would seem remarkable if every demonstrable unit always possessed the same activity. This would be a phenomenon unique in experience with the biological properties of viruses. When all the factors of possible influence are considered, it must be concluded that the results of the present work reveal a remarkably close proportionality of the particle count and enzymatic activity.

Summary

Studies were made of the relationship of the enzymatic dephosphorylation of adenosine triphosphate and the concentration of the specific particulate component of plasmas from chicks with avian erythromyeloblastic leukosis. The results with plasmas from chicks of 19 to 31 days of age revealed a close proportionality between the enzyme and the particles. The relation was more variable with the plasmas of low particle content in which particle counting was most difficult, but became progressively closer with increase in the number of particles. Plasmas from older chicks, 34 to 80 days of age, yielded results of wider variation in some instances. There was no major difference between the relationships observed in the presence of added calcium, which activates the enzyme, and those where no calcium was added. These findings demonstrating the proportionality of the enzyme and the particles in their development in the plasma of chicks during the onset of the disease provide a strong point of evidence that the enzymatic activity may be an intrinsic property of the particles.

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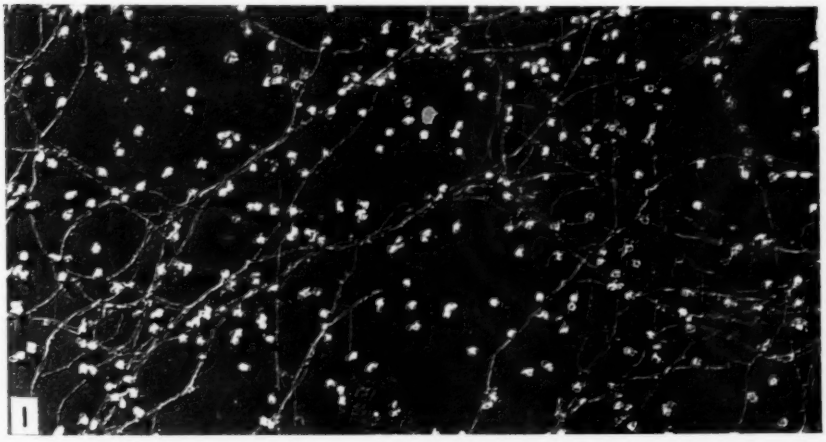
PLATE

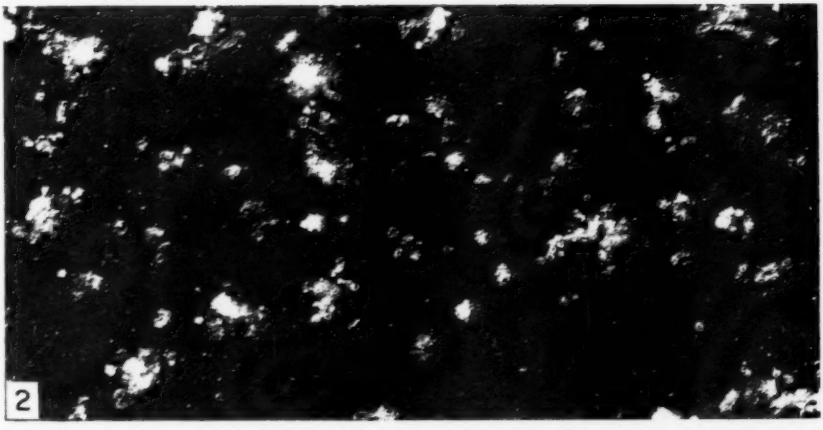
PLATE 81

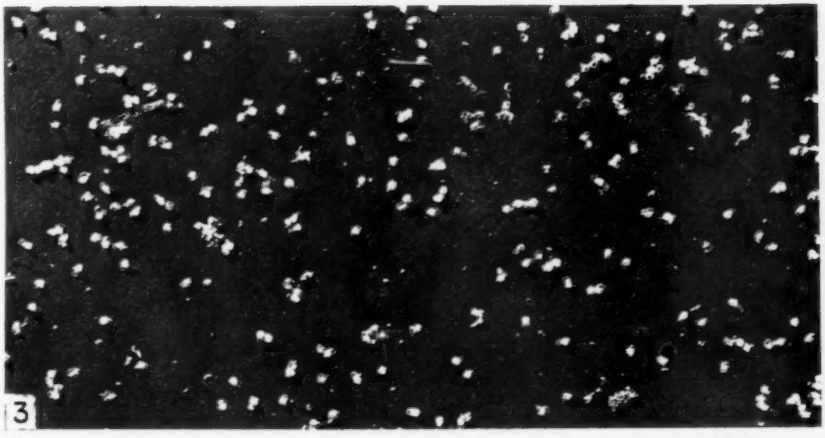
FIGURE 1.—Electron micrograph of a preparation of filtered plasma showing the presence of fibrin strands. × 12,250

FIGURE 2.—Electron micrograph of the sediment from a plasma of low particle content (dilution 1:100) illustrating the effect of the amorphous material in obscuring the specific particles. × 12,250

FIGURE 3.—Electron micrograph of the particles from a plasma of high particle content (dilution 1:2,000) showing the absence of amorphous interfering material. × 12,250







286842—54——2

Virus of Avian Erythromyeloblastic Leukosis. II. Electrophoresis and Sedimentation of the Plasma Particles and the Enzyme Dephosphorylating Adenosine Triphosphate [1,2]

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The initial report of this series (*1*) described the relationship between the specific particulate component (*2*, *3*) of the plasma of chicks with erythromyeloblastic leukosis (*4*) and the enzymatic activity (*5*, *6*) to dephosphorylate adenosine triphosphate. The relationship, apparently linear, was close over the whole range of different levels of particle content and enzyme activity, becoming increasingly so with the more highly active plasmas. The close proportionality of particles and enzyme occurring simultaneously in the plasma during the development of the disease in the host was regarded as evidence that the particles are the source of the enzyme.

Further investigations have been made of the relationships of these two attributes of the plasmas from birds with this disease. Studies have been made of the respective electrophoretic mobilities of the particles and the enzyme in the Tiselius apparatus, and of their rates of sedimentation in the ultracentrifuge. The results obtained by electrophoresis and sedimentation are the subject of this report.

Materials and Methods

Each experiment on electrophoresis of the particles and the enzyme was made with the plasma from a single host. The strain of chicks employed, the procurement of the blood, the removal of the cellular elements, and the preparation of the plasma samples were the same as those already described (*1*) except for the saline medium employed for dilution before filtration through celite and the Selas filters. This dilution was made by mixing equal parts of plasma, 1.5 to 2.5 ml., with the buffer solution to be employed for electrophoresis. After filtration, a further 5-fold dilu-

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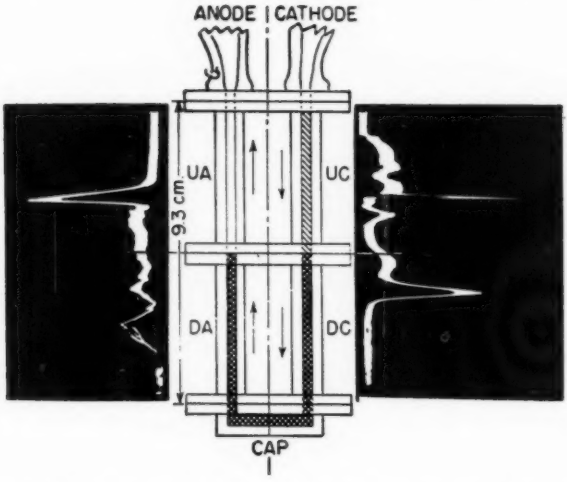
[3] Scholar in Cancer Research of the American Cancer Society, Inc.

tion (total dilution 10-fold) was made with the buffer solution, and dialysis against the same buffer was carried out in the cold for 6 to 8 hours except in one instance, plasma R 906 (see table 1), which was not dialyzed before electrophoresis. These high dilutions were necessary to give sufficient volumes for an experiment with the plasma from a single chick, and they had the advantage of minimizing those electrophoretic anomalies which are the result of high protein concentration.

The experiments were made in the Tiselius apparatus employing a standard 2-part cell as indicated in text-figure 1. The entire cathode limb, upper cathode (*UC*) and down cathode (*DC*) at the right, and the bottom cap (*Cap*) were filled with the diluted plasma. Upon electrophoresis, migration of all observable plasma components proceeded toward the anode, and in all experiments the most advanced component (albumin) was moved well into the next channel, that is, far into *DC* on the descending side and a comparable distance into *UA* (upper anode) on the ascending side (text-fig. 1). The approximate terminal conditions reached in the experiments at *p*H 8.5 with respect to the visible boundaries are illustrated in text-figure 1, in which the ascending and descending diagrams of normal chick serum diluted 1:2.3 are superimposed on a diagram of the electrophoresis cell. It was the object in these studies to bring the migrating boundary of the particles or of the enzyme or both as nearly as possible to the junction of the upper and the lower halves



TEXT-FIGURE 1.—Diagram of the Tiselius cell showing the conditions of an experiment at *p*H 8.5. At the beginning the plasma filled the upper cathode channel (*UC*, single-hatched area), the lower cathode (*DC*) and the *Cap* (cross-hatched areas). During migration the particles and the enzyme were moved out of *UC* and into *DA* (down anode) with the object of shearing the preceding and the following boundaries of the particles or the enzyme or both at the plane between *UA–UC* and *DA–DC*. At the right and left are superimposed the diagrams of normal chicken serum to show the locations of the protein components at the time of shearing.

of the cell, the conditions for which were determined in preliminary studies. When this was realized, the current was turned off and the *U* tube was slipped, isolating the fluid in each of the 5 sections. The contents of each section were then removed after thorough mixing. Dialysis of the electrophoretic fractions was necessary before measurement of the enzymatic activity, since the titrimetric method used was dependent on *p*H change in the reaction mixture. This dialysis was carried out against Ca-free Ringer's fluid at 2 to 4° C. for 12 to 15 hours before samples were taken for the analyses and the particle counts.

Analyses were made at *p*H levels 8.5, 7.5, 6.5 and 6.0. Barbital buffer was used at *p*H 8.5; at all other *p*H levels, phosphate buffer was employed. The ionic strength was 0.1 in all experiments. The electrical field strength applied ranged from 2.7 volts per cm. in some experiments to 6.5 volts per cm. in others in which greater speed of migration was desirable.

In the sedimentation experiments, the plasmas, freed of cells, were diluted with an equal part of Ca-free Ringer's solution before celite and Selas filtration. The plasma, without further dilution, was placed in a specially designed lucite cell (*7*) with 3 holes 2 cm. high and 1/8 inch in diameter. This cell, inserted in the standard rotor of the air-driven analytical ultracentrifuge, was spun at 7,488 × g for various lengths of time [allowance for the times of acceleration and deceleration was made as described before (*7*)], and all fluid above a plane about 2/3 of the way down the cell was carefully removed with a 27-gauge needle 14 mm. long fitted to a 1-ml. syringe. The volume thus taken off (about 0.1 ml.) was determined accurately by the difference in the weight of the cell before and after removal of the fluid. The fluid taken off was washed into 20 ml. of Ca-free Ringer's solution, and measurements were made of the particle content and enzyme activity.

The method for counting the particles and for measurement of enzymatic activity were the same as those used in the work already reported (*1*). Particle counts were made with suitable dilutions of the electrophoretic fractions and the fluid in the 20-ml. volume in the sedimentation experiments. Enzyme studies were made on appropriate volumes of the fluids from the electrophoresis cell sections and from the centrifuge cell to which there were added 0.2 ml. of 1.0 *M* $CaCl_2$ and sufficient Ca-free Ringer's solution to make the total volume to 19.0 ml. (*1*) before the addition of 1.0 ml. of the adenosine triphosphate solution.

Results

The data obtained by electrophoresis are given in table 1, which shows the particle counts and enzymatic activities of the respective fractions in the different experiments.

The results of the particle counts are cited as the number found per ml. of each of the respective fractions, and enzyme activity as gamma of phosphorus liberated in 1 minute by 1 ml. of the same fluid. From these data concerned with particles, it is evident that in certain instances particle concentrations were very similar in the 3 lower sections, *DC*, *Cap*, and *DA*

TABLE 1.—*Distribution in electrophoretic fractions (see text-figure 1) of plasma particles and of the enzymatic activity of the plasma to dephosphorylate adenosine triphosphate*

Plasma	pH	Particle number* (× 10^9)					Enzyme activity† (γ phosphorus)					Mobility (× 10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1})		Mobility ratio (percent)
		UC	*DC*	*Cap*	*DA*	*UA*	*UC*	*DC*	*Cap*	*DA*	*UA*	Particles	Enzyme	P/E
S 922	8. 5	0	214	254	248	52	0	11. 5	12. 1	11. 6	0. 5	−4. 5	−4. 0	
S 636	8. 5	86	258	320	265	1. 1	1. 4	13. 4	15. 3	12. 1	0	−4. 2	−4. 3	
T 198	8. 5	127	674	677	611	0	2. 3	22. 0	22. 0	17. 6	0	−4. 3	−4. 1	
T 15	8. 5	36	244	254	224	0	0	7. 7	9. 8	8. 1	0	−5. 0	−5. 7	
W 343	8. 5	117	724	729	550	1. 4	.....	.....	.....	.....	.....	−4. 3	.....	
											Average.....	−4. 5	−4. 5	100
R 379	7. 5	8	293	282	288	80	0	19. 5	22. 0	20. 5	2. 8	−4. 6	−4. 5	
R 906	7. 5	45	134	132	112	1. 3	3. 8	15. 9	15. 9	12. 4	0	−4. 6	−4. 6	
S 612	7. 5	.....	.....	.....	.....	.....	2. 3	14. 5	18. 2	13. 9	0	.....	−5. 3	
S 930	7. 5	7	561‡	561‡	561‡	10	0	34. 4	40. 9	35. 1	2. 0	−4. 2	−4. 4	
T 259	7. 5	10	142	135	146	19	.....	.....	.....	.....	.....	−4. 9	.....	
T 422	7. 5	45	172	206	204	3	.....	.....	.....	.....	.....	−4. 8	.....	
											Average.....	−4. 6	−4. 7	98
W 183	6. 5	156	725	712	487	66	5. 5	57. 3	52. 4	48. 3	2. 0	−3. 6	−4. 1	
W 469	6. 5	24	348	355	348	24	.....	.....	.....	.....	.....	−3. 9	.....	
											Average.....	−3. 8	−4. 1	93
W 410	6. 0	32	323	547	523	78	1. 9	19. 8	25. 3	22. 8	8. 2	−3. 7	−3. 5	
Q 401	6. 0	99	817	929	1, 090	135	.....	.....	.....	.....	.....	−3. 0	.....	
											Average.....	−3. 4	−3. 5	97

*Per ml. of the fraction.

† Gamma of phosphorus liberated by 1 ml. of fraction in 1 minute.

‡ In all three lower sections an unaccountable aggregation occurred during dilution for counting. Good photographs were obtained with both upper sections so that mobility was calculated from the values derived from these pictures together with the average of the less accurate counts of the lower sections.

(down anode), and that the number of particles seen in the 2 upper sections, *UC* and *UA*, were small or no particles were detected. This result indicates shearing very close to both ascending and descending boundaries of the particles. It should be emphasized that an actual boundary could not be seen because of the low total concentrations of the particles. In other experiments the number of particles in *DA* was smaller than that in the *Cap* and *DC*, but in these cases there were many particles in *UC*, results which show that shearing was not at the boundary but slightly ahead of it as it moved from *UC* into *DC*, and from *DA* into *UA*. These relations were reversed in the other experiments.

Essentially comparable results were obtained in the enzyme studies. As will be discussed later, the ratios of particles to enzyme activity in *UC* and in *UA* tended to be larger than the corresponding ratios for *DC*, *Cap*, and *DA*. The ratios that could be calculated are given in table 2. In 2 cases, S 930 and W 410, however, the *UA* ratios were smaller than those of *DC*, *Cap*, and *DA*. Further information is obtained from the ratios of the enzyme activities of *UC* to the *Cap*, and of *UA* to the *Cap*, in each experiment. This is shown as the "potency ratios" also in table 2 where the average values of *UC*/*Cap* and *UA*/*Cap* are 1.8 and 2.1 respectively. These values emphasize the similarity of the mobilities of the particles and the enzyme and, both being greater than unity, indicate that the enzyme activities, in comparison with the number of particles, of both *UC* and *UA* are less, on the average, than the activities of the fractions *DC*, *Cap*, and *DA*.

Mobilities of the particles and the enzyme were calculated from the data of table 1 in the following way. The distance *Dc* moved in the cathode limb of the cell (*UC* + *DC*) is given by the equation:

$$Dc = 9.3 \frac{[1-(UC + DC)]}{2Ac} \text{ cm.}$$

in which 9.3 cm. = total length of cell; *UC* = concentration of particles or enzyme in fraction *UC*; *DC* = concentration of particles or enzyme in fraction *DC*; and *Ac* = the calculated [4] average number of particles or enzymatic activity in the *Cap*. Correspondingly, the distance moved in the ascending limb is:

$$Da = 9.3 \frac{(UA + DA)}{2Ac} \text{ cm.}$$

From the average of two distances, corresponding field strengths, and times of operation, the mobilities were calculated for table 1. While these values in the various experiments reveal some variation, it can be said that, with consideration of the materials and methods employed, this variation seems exceedingly small. The averages of the mobilities of the enzyme and of the particles in the respective experiments were nearly identical at

[4] In order to arrive at a number based on the particle counts made on all five of the fluids in a given experiment the *Ac* value was taken as the sum of the particles in all five limbs divided by 3.

calculated from the values derived from these pictures together with the average of the less accurate counts of the lower sections.

TABLE 2.—*Ratios of particle counts of table 1 (without factor 10^9) to enzyme activity given in table 1 (gamma of phosphorus liberated by 1 ml. of fraction in 1 minute)*

Plasma	pH	Ratio of particles to enzyme activity					Potency ratio*	
		UA	*DA*	*Cap*	*DC*	*UC*	*UC/Cap*	*UA/Cap*
S 922...	8. 5	104	21. 4	21. 0	18. 6	.....	.....	5. 0
S 636...	8. 5	.....	21. 9	20. 9	19. 3	61. 4	2. 9	.....
T 198..	8. 5	.....	34. 7	30. 8	30. 6	55. 2	1. 8	.....
R 379..	7. 5	28. 6	14. 0	12. 8	15. 0	.....	.....	2. 2
R 906..	7. 5	.....	9. 0	8. 3	8. 4	11. 8	1. 4	.....
S 930...	7. 5	5	16. 0	13. 7	16. 3	.....	.....	. 36
W 183..	6. 5	33	10. 1	13. 6	12. 7	28. 4	2. 1	2. 4
W 410..	6. 0	9. 5	22. 9	21. 6	16. 3	16. 8	. 78	. 44
						Average.....	1. 8	2. 1

*These values are the ratios of the number of particles in *UC* and *UA* with respect to those of the *Cap* relative to unit dephosphorylation of adenosine triphosphate, and were derived as the quotients of the respective ratios of particles to enzyme activity given in the preceding columns of this table.

TABLE 3.—*Distribution of particles and enzyme in plasma after various times of ultracentrifugation at 7,488 × g*

Exper. No.	Plasma	Time (*min.*)	Particles* ($\times 10^9$)	Enzyme activity† (γP)	Percent of control‡	
					Particles	Enzyme
1	T 426	Control	160	53	100	100
		20. 1	126	50	79	94
		37. 6	65	26	41	49
		49. 7	51	19	32	36
2	T 729	Control	238	180	100	100
		20. 6	190	115	80	64
		35. 8	68	75	29	42
		49. 8	52	61	22	34
3	Q 638	Control	270	93	100	100
		19. 9	187	64	69	69
		43. 4	113	39	42	43
4	U 531	Control	312	.....	100	.....
		62. 1	30	.....	10	.....
5	Q 958	Control	710	277	100	100
		31. 0	231	80	33	29
		58. 2	71	18	10	7
6	Q 946	Control	665	.....	100	.....
		52. 7	36	.....	5	.....
7	X 49	Control	992	.....	100	.....
		50. 6	69	.....	7	.....

* Particles per ml. of the control plasma and of the plasma removed from the cell.

† Gamma of phosphorus liberated by 1 ml. of the control plasma and of the withdrawn plasma in 1 minute.

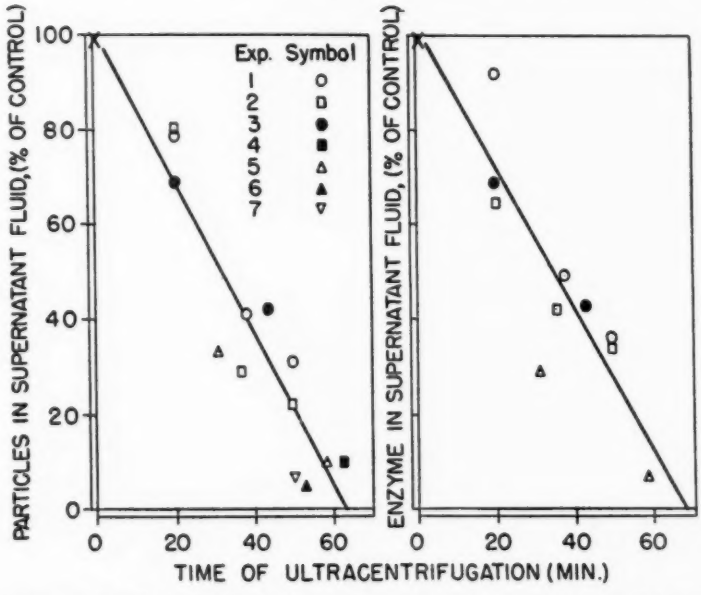
‡ Percent of particles or enzyme in the control and those remaining in the fluid withdrawn from the cell after the various periods of centrifugation.

all *p*H levels, the variation on the whole being no more than 7 percent. It is notable that the mobilities became progressively smaller with decrease in *p*H below 7.5, and this occurred proportionally with both enzyme

and particles. It is of interest, further, that *p*H 6.5 appeared to be still well above the isoelectric points of the particles and the enzyme.

The results of the sedimentation experiments are summarized in table 3 and text-figure 2. In table 3 are given the particle counts made on the fluids withdrawn from the centrifuge cell after different intervals of spinning 7 plasma specimens, together with analogous data obtained by determination of enzyme activity on the same samples. The values given indicate the number of particles per ml. of the fluid taken from the cell, and enzyme activity in terms of gamma of phosphorus liberated by 1 ml. of this fluid in 1 minute. Enzyme activity was measured in 4 of the 7 experiments. The particle counts in the other 3 experiments were made in connection with similar studies on the infectious unit reported in another paper (*8*). For purposes of intercorrelations and simpler comparisons, the data are presented also as the percentages given in table 3.

A clearer view of the relationship between the sedimentation properties of the particles and those of the enzyme may be obtained by inspection of text-figure 2. To the left, the numbers of particles remaining in the withdrawn fluids are plotted against the time of centrifuging these various samples, and to the right there is a similar comparison of enzyme activity with the period of centrifugation. The scatter of points about the lines, drawn through the data by the method of least squares, is at



TEXT-FIGURE 2.—Comparison of the rates of sedimentation of the particles and of the enzyme activity. The numbers of the experiments correspond to those of table 3. The slopes of the lines, drawn through the points by the method of least squares, were −1.59 for the particle relationships and −1.47 for those of the enzyme activity. The respective intercepts on the abscissae were 63 and 68 minutes.

once evident. This was due largely, if not entirely, to variations in the viscosity and density of the different plasmas, and the effects of these are included in the data. The volumes were too small for direct viscosity measurements under the conditions of the experiments, but differences great enough to account for the scatter of the points were observed in studies on other plasmas.

It should be emphasized that the data have been presented in the manner of text-figure 2 principally for convenience. The relationship between particles and enzyme are clearer on comparison of the particle count on a given sample with the enzyme activity determined on the same sample. When this is done by inspection of the respective pairs of symbols of text-figure 2, it is seen that the relationship is indeed close.

This is further evident in the consideration of the slopes of the lines, which were -1.59 for the particles and -1.47 for enzyme activity, and the intercepts of the 2 lines extrapolated on the time coordinates. The latter show that the period of spinning that would be required for sedimentation of the particles beyond the level of sampling in the ultracentrifuge cell is 63 minutes, and that for the enzyme is 68 minutes.

Discussion

Quantitative evidence of the occurrence in proportional amounts of the specific particulate component and of the enzyme dephosphorylating adenosine triphosphate in the plasma of birds with erythromyeloblastic leukosis has suggested (*1*) that the enzyme activity is a part or a function of the particles. In the present work two other criteria have been applied as a basis for testing the relationship between particles and enzyme. One of these was a series of experiments with electrophoresis to learn whether the particles and the enzyme could be separated by virtue of differences in electrical charge, and thus of differences in rate of migration in an electrical field. The second was concerned with detection of any differences that may exist between the rates of sedimentation of the particles and the enzyme, properties dependent on the size, shape, and density of the units of the two materials.

It is evident from the data given here that the respective rates of migration of the particles and the enzyme are essentially identical within a range of 7 percent variation in the single experiment at *p*H 6.5, and 3 percent or less in the remainder at the *p*H levels from 8.5 to 6.0. This identity was maintained as the mobility decreased progressively in the successive steps from the alkaline to the acid region. It is notable, as would be expected, that variations in the results were greater in the individual experiments than those reflected by the average values at the different *p*H levels. It is likely that much of this was due to variable stability of the enzyme in the most dilute fractions [5] (dilute with respect to both enzyme and plasma protein) during the periods of electrophoresis and subsequent prolonged dialysis. This was seen (table 1) in all except two, S 930 and W 410, of the experiments in which the particles of both

[5] This has been demonstrated in other experiments to be reported by Dr. Irving Green.

UC and *UA* were associated with a smaller proportion of enzyme activity than those of the *Cap* section. The reverse was true in experiments S 930 and W 410.

The variation, summarized in table 2, was random, therefore, with respect to the data with both *UC* and *UA*, a fact of critical importance in the interpretation of the results. For example, had the number of particles per unit of enzyme activity always been greater in *UA* than in *UC*, the finding would have indicated a mobility of the particles greater than that of the enzyme. That this was not the case is clearly seen in table 2, in which the data are summarized in the ratios of particles to enzyme activity, and in the relative numbers of particles per unit enzyme activity in *UA* and *UC* as compared with the attributes of the materials in the *Cap*. It should be emphasized that the method used here provides a very sensitive means for comparing the mobilities of two stable materials. Even under the difficult conditions of the present experiments, it can be concluded from the mobility ratios given in table 1 that a possible difference in the mobilities of the particles and the enzyme was not greater than 7 percent.

The experiments with the ultracentrifuge showed that the enzyme activity is carried by a particulate material of the same sedimentation rate as that of the specific particles in the plasma. If the two populations of particles were of the same shape and density, a possible difference in average diameter would be no greater than 3 percent.

By consideration of the present work and that reported elsewhere (*1*, *5*), it can be concluded that: 1) the enzyme and the specific particulate component occur proportionally in the plasma of chicks developing erythromyeloblastic leukosis; 2) both attributes are inseparable by electrophoresis at *p*H 8.5 to 6.0; 3) both sediment at the same rate in the ultracentrifuge; and 4) in the ultracentrifugal purification process, the enzyme follows the particles. From these data it is difficult to evade the hypothesis that the enzyme is carried by the particles. Otherwise, these quantitative relationships constitute a remarkable series of coincidences. Although it seems established that it is a part of the particle, the origin of the enzyme and its physical or chemical relation to the particle are problems for further study. The question of enzyme adsorption is an ever-present one that has troubled the concepts concerned with other viruses, so much so that doubt has been expressed of the possibility of demonstrating an enzyme as an integral part of the virus particle (*9*). The earlier assumption (*2*, *3*) that the particulate component is the virus of erythromyeloblastic leukosis has been well substantiated in work described in an accompanying report (*8*) and, consequently, those doubts pertaining to viruses are applicable in general to the agent of this form of leukosis. A very considerable difference between the case with this agent and that of the viruses previously studied (*9*), except the influenza virus (*7*), are the facts that 1) the plasma of normal chicks contains no measurable enzyme, and 2) *all* of the enzyme always follows the particles. The enzyme likewise follows the infectious qualities of the plasma (*8*), both accompanying the particles. It must be

concluded, therefore, either that, as discussed before (*5*), the enzyme is an integral part of the particle, or that adsorption of the enzyme must have occurred within the cell from which the particle presumably was liberated into the plasma. Whatever the relationship should eventually prove to be, it is already certain that it is an intimate one.

Summary

Studies were made on the electrophoresis of the enzyme dephosphorylating adenosine triphosphate and of the specific particulate component in the plasma of young chicks with erythromyeloblastic leukosis. In the Tiselius apparatus, both enzyme and particles migrated to the anode at essentially identical rates in 9 experiments at the *p*H levels of 8.5, 7.5, 6.5, and 6.0. The greatest difference in the average rates of migration of the two materials was 7 percent in one experiment, and no greater than 3 percent in the remaining groups. The rate of migration of these particles progressively decreased from -4.5 ($\times 10^{-5}$ cm.^{2} sec.$^{-1}$ volt^{-1}) at *p*H 8.5 to -3.4 at *p*H 6.0, and of the enzyme from -4.5 to -3.5 over the same *p*H range.

Ultracentrifugation of similar plasma specimens showed that the particles could be sedimented from the portion of the suspension tested in 63 minutes and the enzyme activity in 68 minutes, a difference that was not significant.

The results were interpreted as showing that the enzyme activity is carried by particles of the same sedimentation characters as the specific particulate component and that the two are inseparable by electrophoresis. Earlier work has shown that the two attributes occur simultaneously and proportionally in the plasma of young chicks developing the disease, and that the particles and enzyme remain associated in the centrifugal purification of the specific particles. These consistent correlations have led to the conclusion that the enzyme is a part or a function of the particulate component.

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Virus of Avian Erythromyeloblastic Leukosis. III. Interrelations of Plasma Particles, Infectivity, and the Enzyme Dephosphorylating Adenosine Triphosphate [1, 2]

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Quantitative relationships have been established between a specific particulate component (*1*, *2*) and an enzyme (*3*) dephosphorylating adenosine triphosphate present in the blood plasma of chicks with erythromyeloblastosis (*4*). A close proportionality between enzyme and the particles counted in electron micrographs was found (*5*) in the plasma as the chicks developed the disease. The particles and the enzyme were inseparable by electrophoresis (*6*) and both sedimented (*6*) at the same rate in the ultracentrifuge.

The plasma from the same birds contains, likewise, the viral agent transmitting the disease, and it had been assumed (*1*, *2*) earlier that this infectious unit was identical with the particulate component. In view of the established relations between the particles and the enzyme, however, it has seemed imperative to attempt a demonstration of whether or not the particles carry not only the enzyme activity but the property of infectiousness as well. In the present work, therefore, an investigation has been made of the relation of the infectious unit to the enzyme and to the particles in its occurrence in the plasma; in its behavior on electrophoresis; and in its sedimentation properties in the ultracentrifuge. The results of this work are given in the present report.

Materials and Methods

The origin of the strain of erythromyeloblastic leukosis used in this and all of the other studies of the disease in this laboratory, and the pathological aspects of the condition, have been described elsewhere (*7*). The source of the donor chicks providing the plasmas, the preparation of the plasmas for study, the method of counting the particles in the plasma and of measuring enzyme activity, as well as the procedures for electrophoresis

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and ultracentrifugation, were the same as those employed in the studies on the particles and enzyme already described (*5*, *6*).

It should be emphasized that all of the plasmas were filtered, principally for the purpose of removing as nearly as possible all of the primitive cells which, themselves, can transmit the disease. The filtration process was carried out, after plasma dilution of 1 volume to 1, or 1 volume to 2 volumes of the appropriate saline solution, through a mat of celite #512 and small Selas filters. The latter, MFI–11 (Selas Corporation of America, Scientific Equipment Division, Philadelphia, Pa.), were of porosity #02, rated by the manufacturer as "having a maximum pore diameter of 0.85 micron—fine enough to effect a complete sterilization even where the solution is heavily contaminated." In tests with *Escherichia coli*, however, the filters were not absolutely bacteria-tight, a few bacterial cells traversing the disk when undiluted cultures of the organisms were used. It seemed improbable, nevertheless, that the few primitive cells possibly passing such filters could exert any significant influence on the infectivity of the plasmas.

The experiments were made in 3 groups: 1) titration of infectious capacity on filtered but otherwise untreated plasmas, on which enzyme measurement and particle counts were made; 2) titration of electrophoretic fractions in association with particle counts; and 3) titration of ultracentrifugal fractions together with particle counts. In the latter two categories all three determinations could not be made on the same plasmas because of the limited amount of material or the number of test chicks available.

1) Blood for these studies was obtained from chicks, selected the day before the experiment by the micro screening test (*8*), by heart puncture for the 4 specimens of experiment 78, table 1, and in all other instances by section of the saggital sinus. Heparin was used in the ratio of about 10 mg. per 10 ml. of blood; in experiments 78, 80, and 81, the heparin solution (Liquaemin sodium, Organon, Inc., Orange, N. J.) contained 10 mg. per ml., and in experiments 84, 91, 92, 93, and 94 the preparation from the same source contained 50 mg. heparin per ml. The change was made to avoid excessive dilution of the smaller volumes of plasma and to lessen the amount of phenol, used as preservative in the commercial heparin solution.

Counts were made of the primitive cells (white blood cells) on specimens taken immediately from the thoroughly mixed, heparinized blood samples. The diluent was a solution of phloxin (*9*) in calcium-free Ringer's solution, and the counts were made in duplicate in Neubauer bright-line chambers. The first centrifugation of the chilled blood was made in pointed, calibrated centrifuge tubes, giving an estimate of the relative proportions of plasma, primitive cells, and red blood cells. The demarcation between the white-cell (primitive-cell) and red-cell layers was usually very indistinct, so that the volumes given in table 1 are but approximations.

The saline diluent used in the filtration process was Ca-free Ringer's solution. The dilution before filtration of plasma S 939 (experiment 80)

and all of the plasmas of experiment 84 was 1 volume of plasma to 2 volumes of the saline solution; in all other instances, the proportions were 1 volume of plasma to 1 volume of saline. Samples of the plasmas were taken immediately after filtration for particle count and enzyme measurement. Further dilutions of the samples to be titrated were made with BSA-Simms' solution (*9*). The initial dilution of the filtered plasma was made by adding 1 volume of plasma to 4 volumes of BSA-Simms' solution in experiments 78, 80, 81, and 84. In the remainder, 1 volume of plasma was diluted with 9 volumes of BSA-Simms' solution. These materials constituted in each case the initial inoculum injected into chicks. Two further dilutions were made in 10-fold intervals to give a total of 3 different dilutions of each material for inoculation into the test chicks.

The test chicks, like the donor birds, were White Leghorns of line 15 (*10*) obtained from the Regional Poultry Research Laboratory, East Lansing, Michigan. A laying flock of these birds was maintained and the eggs were hatched in the laboratory. The chicks were inoculated intravenously at 3 days of age as described before (*9*), and blood smears were made each day, except Sunday, for about 14 days during the appropriate period after inoculation, which could be judged on the basis of the expected activity of the inoculum. Smears were made thereafter on alternate days or twice a week for an average total of about 50 days from the time of inoculation. Each dilution of each material was inoculated into 30 to 35 test chicks. The data were analyzed on the basis of the latent period by procedures described in another report by Eckert *et al.* (*11*). In the present paper, it suffices to state that the level of infectivity or degree of infectiousness is given in terms of latent period in log days. From each material inoculated, a point was obtained for each dose, and the three points were plotted for locating the regression line which was given the slope of the average of the individual slopes of all of the plasmas studied in this series. The point on this line selected for designating the level of infectivity was that corresponding to the result, in log days, found or expected with the inoculum containing 10^{-3} ml. of the initial whole plasma. This dose was approximately the mean value for the individual plasmas.

2) Electrophoretic fractions for particle count and titration were obtained by the same techniques used (*6*) in the comparison of particle count with enzyme activity, with a single difference. Whereas it had been necessary to dialyze the fractions for the enzyme studies (*6*), this could be omitted in the titration of infectivity. Appropriate dilutions of the fractions were made with BSA-Simms' solution, and injections into chicks were carried out as before.

3) In the sedimentation studies carried out by the techniques already described (*6*), the number of titrations possible with a given sample of plasma was restricted principally by the limitation of the method of titration. In the comparison of particle count with enzyme activity, three periods of centrifugation were employed (*6*). The experiments with virus infectivity were designed to provide a control material with no

activity removed and one centrifuged material with nearly all of the virus sedimented. It had been intended to retain in the supernatant fraction about 10 to 20 percent of the infectivity; in practice, as seen later, less than this remained.

Results

Infectivity, particle count, and enzyme activity were investigated in a series of 31 separate plasmas, resulting in the data given in table 1. An attempt was made in every experiment, using from 3 to 5 plasmas depending on the number of test chicks available, to select specimens of infectivities of widely variable levels as judged by the micro screening test (*8*). A good selection was not always available since the choice was limited by the donor chicks at hand on the day the test chicks became 3 days of age. As seen in table 1, however, the group represented a fairly wide range of 1.5 to more than 15 minutes micro-reaction time. Inspection of table 1 shows, likewise, a relatively wide variation in the latent period of the disease in the various plasma donors and in the ages of the donors (age at bleeding plus 3 days) and the duration of the disease before blood was taken.

The observed data relative to level of infectivity (expressed as log latent period in days), to particle count (the number of particles per ml. of the original plasma), and to enzyme activity (gamma of phosphorus liberated by 1 ml. of the plasma in 1 minute) are given in table 1. One of the plasmas, O 935, in experiment 78, failed to infect chicks in the period of study. Analysis of these data showed that the relationship of particles to enzyme activity was not absolute throughout the period of the experiments, namely 4 months. Thus, the relations between particles and enzyme did not fall within the limits of variation reported in the initial paper (*5*) of this series which described work carried out in the short period of 7 weeks. It was observed, however, that in any single experiment, as can be seen from the data of table 1, the particle count was related to enzyme activity; and consequently, it was possible to make the comparison of infectivity, particle count, and enzyme activity in each experiment separately. The data were then brought together to show the interrelations in the various experiments. These are revealed in text-figure 1. The chart at the left shows the relation of infectivity to particle count and that to the right the relation of infectivity to enzyme activity. The lines were drawn with the average slope of all of the infectivity measurements to pass through the mean infectivity level derived from the experimental observations of table 1. The mean infectivity level and particle count or enzyme activity for each experiment was fitted to the line, and the analogous points representing the data for each plasma of a given experiment were adjusted about the lines on this basis.

After these calculations, it is seen that the individual points, one for each plasma, are scattered in an orderly fashion about the lines in both graphs, indicating an unequivocal relationship between infectivity and particle count, and infectivity and enzyme activity. It is apparent also that the characters of the relationships are the same in both instances.

TABLE 1.—*Data obtained in the study of the interrelationships of the infectious capacity, activity to dephosphorylate adenosine triphosphate, and the particle content of the plasmas from 31 chicks with erythromyeloblastic leukosis*

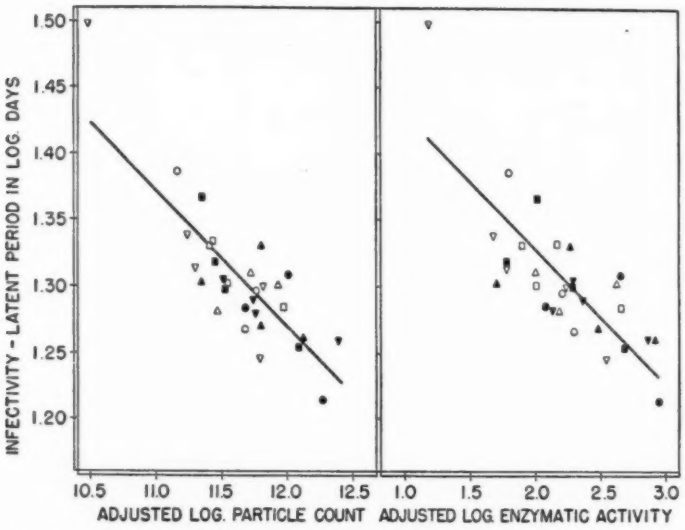
Exp. No.	Donor chicken	Latent* period (*days*)	Age bled (*days*)	Plasma volume (*ml.*)	WBC volume (*ml.*)	RBC volume (*ml.*)	WBC count (*mm.*^{3})	Micro† test (*min.*)	ATPase‡ activity (γ *P*)	Particle‖ count ($\times 10^9$)	Log latent period (*days*)
78	R 420	34	55	13. 8	.....	.....	690, 000	5	312	347	1. 267
	O 406	46	61	7. 2	.....	.....	1, 430, 000	10	264	420	1. 296
	O 935	47	55	9. 5	.....	.....	790, 000	15	1. 4	6. 6	>1. 5
	O 139	57	69	12. 2	.....	.....	580, 000	15	98	105	1. 386
80	O 354	43	77	2. 5	6. 9	.....	925, 000	3	716	806	1. 284
	S 365	39	43	3. 9	9. 1	1. 0	1, 215, 000	5	124	218	1. 330
	S 939	12	16	1. 3	1. 3	0. 2	1, 100, 000	9	194	233	1. 315
	S 757	22	31	2. 8	2. 9	0. 7	900, 000	11	161	275	1. 302
81	T 50	16	23	2. 5	3. 4	0. 6	1, 335, 000	5	661	1043	1. 302
	S 842	19	23	2. 8	3. 1	0. 9	1, 105, 000	10	232	351	1. 281
	T 62	16	23	2. 8	1. 7	0. 4	1, 020, 000	14. 5	162	648	1. 310
84	W 188	14	20	2. 2	2. 5	0. 6	1, 520, 000	1. 5	223	960	1. 281
	T 451	27	35	3. 2	0. 8	1. 2	330, 000	12	80	281	1. 296
	W 19	18	27	2. 3	5. 0	1. 2	1, 210, 000	3. 5	456	850	1. 227
	W 78	17	20	2. 3	1. 4	0. 4	1, 090, 000	9	62	251	1. 320
	T 82	37	43	5. 3	3. 3	1. 9	640, 000	15	20	44	1. 479
91	X 805	21	34	12. 5	10. 0	0. 6	1, 905, 000	3. 5	424	1516	1. 214
	X 861	22	34	8. 4	5. 8	0. 7	745, 000	12	57	394	1. 285
	U 143	17	28	7. 7	5. 1	0. 6	1, 470, 000	7. 5	211	843	1. 308
92	U 473	15	21	3. 4	2. 0	0. 5	1, 310, 000	12. 5	119	248	1. 367
	U 496	15	21	2. 1	2. 6	0. 5	1, 680, 000	2. 5	571	1363	1. 254
	U 498	15	21	2. 9	1. 2	0. 5	1, 060, 000	15	73	312	1. 318
	U 268	26	35	2. 9	2. 1	0. 5	1, 320, 000	7	226	379	1. 297
93	X 885	39	48	2. 5	5. 3	0. 7	1, 870, 000	8	156	838	1. 330
	Q 935	28	62	5. 6	9. 3	3. 1	1, 510, 000	15	44	303	1. 303
	U 51	39	48	7. 5	12. 7	2. 3	1, 450, 000	10	249	863	1. 271
	U 13	32	48	5. 8	19. 7	1. 6	2, 300, 000	1. 5	701	1754	1. 262
94	U 915	20	28	3. 0	4. 3	0. 7	1, 350, 000	6. 5	202	389	1. 290
	U 701	22	35	3. 0	6. 9	0. 6	1, 790, 000	2. 5	637	1745	1. 261
	U 617	28	35	4. 4	4. 5	1. 1	1, 500, 000	16	157	241	1. 305
	U 788	19	28	3. 6	5. 0	1. 0	1, 160, 000	12. 5	119	398	1. 280

*Latent period of the disease in the donor bird.

†Micro screening test (*8*) for ATPase activity of plasma made on the plasma of the donor on the day before the bird was bled.

‡Gamma phosphorus liberated by 1 ml. of plasma in 1 minute.

‖Number of particles per ml. of plasma.



TEXT-FIGURE 1.—Relationship of particle number and of enzyme activity to dephosphorylate adenosine triphosphate to the infectivity, stated in terms of the logarithm of the latent period, of the plasmas from chicks with erythromyeloblastic leukosis. The values of particle numbers and enzyme activity are not absolute but were adjusted as described in the text. Each symbol represents the results with a single plasma, and similar symbols indicate the individual plasmas in a given experiment.

The findings encountered in electrophoresis of 5 plasma specimens are given in table 2. Here the amount of material limited the studies to a comparison of the mobilities of the particles with those of the infectious unit in the various *p*H regions. One experiment was made at *p*H 8.5, two at *p*H 7.5, and one each at *p*H levels of 6.5 and 6.0. The values for particle numbers given in table 2 represent the counts per ml. of the different fractions taken from the electrophoresis cell. The infectious titers are stated in relative terms; as a basis for the calculation, the average of the individual infectious levels of the *Cap*, *DC*, and *DA* fractions (*6*) was employed as unit infectivity, and the percentage levels of the infectivity of the separate fractions were determined from this value in each experiment. The mobilities of the particles given in table 2 were included in a previous paper of this series [table 1 of (*6*)]. Calculations of the mobilities of the infectious unit were made in the same way as for the mobilities of particles, employing the percentage values given in table 2.

The results reveal a very close similarity between the mobilities of the particles and those of the infectious unit at the different *p*H levels. The mobilities of the particles in the plasmas studied in this work are essentially the same as the average values obtained in the whole group of experiments [table 1 of (*6*)] at the same *p*H levels. Variation in the mobilities of the infectious unit is somewhat greater, as might be suspected, than those of the particles but are well within the limits of values

TABLE 2.—*Distribution in electrophoretic fractions [see text-fig. 1 of (6)] and mobilities of plasma particles and of the infectious unit present in the plasma*

Plasma	pH	Particle number* ($\times 10^9$)					Infectious titer† (*percent*)					Mobility ($\times 10^{-5}$ cm.^{2} sec.$^{-1}$ volt^{-1})		Mobility ratio (*percent*)
		UC	*DC*	*Cap*	*DA*	*UA*	*UC*	*DC*	*Cap*	*DA*	*UA*	Particles	Infectivity	P/I
W 343	8. 5	117	724	729	550	1. 4	3	132	116	73	0. 2	−4. 3	−3. 9	110
T 259	7. 5	10	142	135	146	19	1. 4	146	78	97	7	−4. 9	−3. 8	111
T 422	7. 5	45	172	206	204	3	14	82	125	101	4	−4. 8	−5. 1	94
W 469	6. 5	24	348	355	343	24	4	77	116	121	13	−3. 9	−4. 9	80
Q 401	6. 0	99	817	929	1090	135	4	112	110	83	16	−3. 0	−2. 4	125

*Per ml. of the fraction.

†Relative infectivities based on the average of the infectivity levels (log latent period in days) of the *Cap*, *DC*, and *DA* fractions.

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expected on the basis of biological measurements of virus infectivity. As in the instances of the particles and the enzyme activity, there is a decrease in mobility of the infectious unit toward the lower *p*H levels without close approach to the isoelectric point.

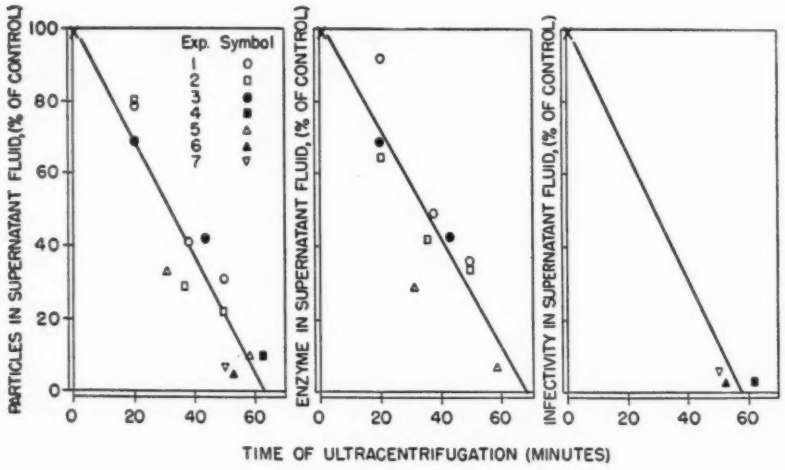
TABLE 3.—*Distribution of particles and infectious unit in plasma after various times of ultracentrifugation at 7,488 × g*

Exper. No.	Plasma	Time (*min.*)	Particles* (× 10^9)	Percent of control†	
				Particles	Infectivity
4	U 531	Control	312	100	100
		62. 1	30	10	3
6	Q 946	Control	665	100	100
		52. 7	36	5	3
7	X 49	Control	992	100	100
		50. 6	69	7	6

*Particles per ml. of the control plasma and of the plasma removed from the cell.

†Percent of particles and infectivity in the control and that remaining in the fluid withdrawn from the cell after the various periods of centrifugation.

The results of the sedimentation experiments are given in table 3. Text-figure 2 shows a comparison of the findings in the present studies on infectivity with those pertaining to the particles and the enzyme already reported (*6*). The portion of the figure at the left shows the sedimentation rate of the particles in a total of 7 experiments, including those (experiments 4, 6, and 7, table 3) of the present work. The central

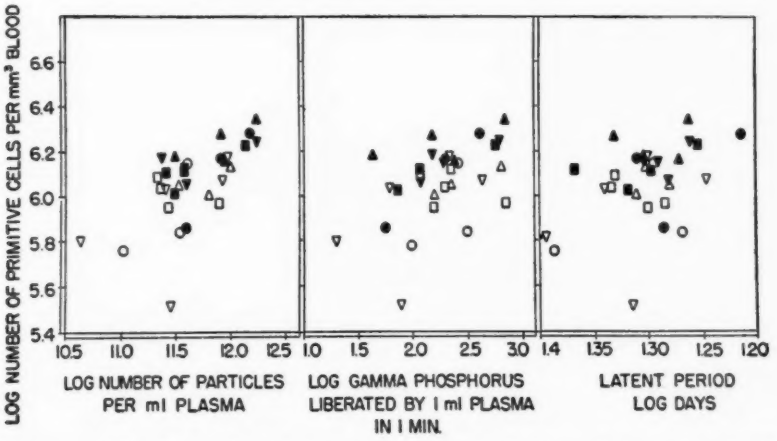


TEXT-FIGURE 2.—Comparison of the rates of sedimentation of the particles (*left*), enzyme activity (*center*), and the infectious entity (*right*). The lines were drawn by the method of least squares, and the intercepts on the abscissae—63, 68 and 58 minutes, respectively—represent the time of centrifugation at 7,488 × g theoretically required to sediment all of the particles, enzyme activity, and infectivity just beyond the fluid level sampled.

portion of text-figure 2 shows the sedimentation characters of the material carrying the enzyme, determined in the 4 experiments reported earlier (*6*). At the right the points indicate the percent of virus infectivity removed by centrifugation of the three plasmas of experiments 4, 6, and 7, the same samples on which the particle counts were made to give the analogous points of the part of the figure at the left. The different symbols correspond to the same experiments in all of the graphs of the figure.

The lines through the points were drawn by the method of least squares. The intercepts at the abscissa, minutes of centrifugation, revealed that the periods which would be required for sedimentation of all of the particles, enzyme, and virus (infectivity) below the level of sampling were 63, 68 and 58 minutes, respectively. Thus, it is evident that the three attributes of the plasmas possess practically identical sedimentation rates within the limits of the method of investigation.

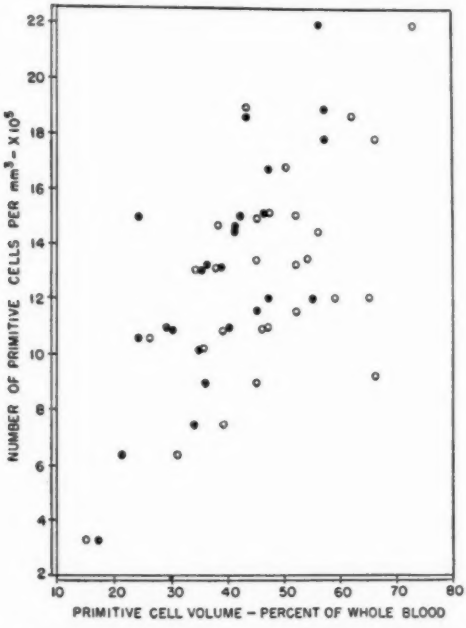
An examination was made of the relationships between the primitive-cell content of the different blood samples and the particles, dephosphorylating enzyme, and infectivity of the plasmas from these same blood specimens. The primitive-cell numbers (table 1) varied through the relatively narrow range of 330,000 (T 451, exper. 84) to 2,300,000 (U 13, exper. 93). In text-figure 3 the number of primitive cells per mm.^{3}, indicated in the ordinate, are compared, at the left, with log particle numbers in the abscissa; in the center graph with enzymatic activity (log gamma phosphorus liberated by 1 ml. of plasma in 1 minute); and at the right with infectivity (log latent period in days). These results indicate a definite trend toward relationship between cell count and the three different entities of the plasma. It should be remembered that this appearance of correlation may be ambiguous; all of the blood specimens were selected for the studies on the basis of dephosphorylating activity and do not represent a random cross



TEXT-FIGURE 3.—Comparison of the concentration of primitive cells in the blood with the number of particles (*left*), enzyme activity (*center*), and infectivity (*right*) of the respective plasmas.

section of the population as a whole. This will be discussed in a later paragraph.

It was of interest in this work to learn something of the means for measuring the primitive-cell content of birds with erythromyeloblastic leukosis in large-scale routine work. A very rough estimate can be obtained from blood smears which is useful in the selection of birds for further study. A better approximation is available from the primitive-cell volume observed in carrying out the micro screening test (*8*), but this is complicated by the lack of sharp separation of the red from the



TEXT-FIGURE 4.—Relation of the number of primitive cells in the blood to the primitive-cell volume (percent of total blood volume) determined by measurements in the micro screening test (see text), indicated by the solid circles, and by sedimentation of the cells in pointed 15-ml. tubes (open circles). In both cases centrifugation was at 1,029 × g for 15 minutes.

white cells. In order to see the extent of the variation to be expected, the primitive-cell counts are compared in text-figure 4 with the hematocrit values available from the micro screening test and those obtained in the separation of plasma from the cells by centrifugation. Assuming that the cell counts are relatively accurate (duplicate counts did not vary more than 10 percent), it is seen that the hematocrit values are quite variable. This was true both with those from the micro test and those resulting from centrifugation of the blood in pointed, graduated tubes of 15-ml. volume. In general, the relative primitive-cell volumes observed in the 15-ml. tubes were greater than those determined by the former means. It

should be emphasized that the values were obtained in routine studies and that no special efforts were made to maintain rigidly the conditions of centrifugation.

Discussion

The purpose of the work described in this series of reports (*5*, *6*), and in the present report, has been the study of possible interrelationships of a specific particulate component, an enzyme dephosphorylating adenosine triphosphate, and the infectious entity contained in the plasmas of birds with erythromyeloblastic leukosis. These relationships have been investigated in a comparison of the three materials 1) as they appear in the plasma of chicks developing the disease following experimental inoculation with the virus; 2) in the comparison of their electrophoretic properties; and 3) in the comparison of their sedimentation behavior. The results have shown that the particulate component and the enzyme activity occur in the plasma with quantitative simultaneity with the onset of the disease, and that the particles and the enzyme activity possess identical electrophoretic mobilities and rates of sedimentation in the ultracentrifuge. These findings provide excellent evidence that the enzyme activity is carried by the specific particles that are seen in the electron microscope.

The evidence of the identity of the particles with the infectious unit is less thorough than that pertaining to the enzyme, due in part, undoubtedly, to the complex factors involved in the biological titrations of the virus. Infectivity of the plasmas was obviously related to both particles and enzyme, as seen in text-figure 1 of this paper, but with greater variation than existed in the comparison of the enzyme and the particles shown by text-figures 5 and 6 of (*5*). It is of interest that the variations in infectivity as compared with the number of particles were similar to those of infectivity with the enzyme. This is plain on inspection of the various symbols in the two graphs of text-figure 1 and further evident in the similar values of the average deviation, ± 0.3 and ± 0.4, derived from the sum of the squares of the deviation from the lines in the two sets of data. In the electrophoretic studies it is seen that, despite the small number of observations, the mobility of the infectious unit was closely similar to the mobilities of the particles and the enzyme. It was in the sedimentation studies that the best correlation was observed; here (text-fig. 2) it was clear that the sedimentation properties of the infectious unit were essentially identical with those of the particles and the enzyme.

The findings enumerated would appear to provide a satisfactory basis for the hypothesis that the specific particulate component seen in the host plasmas with this disease constitutes the infectious unit or virus and bears with it the material exerting the enzyme activity. This is not meant to imply that the enzyme is the infectious moiety of the particle or that the two properties are any more closely related than in the sharing of a common vehicle of existence. Furthermore, while it seems certain that the infectious property and the enzyme go along always with the particles, it may still be said that the specific materials exhibiting the separate prop-

erties are adsorbed on the particles. This has been a possibility cited many times with respect to virus and particles isolated from tissues diseased with other viruses, but one of invariable failure of subsequent confirmation. Adsorption of the enzyme on virus particles is a more plausible supposition, for evidence has been obtained of the implication of enzymes with viruses. The conditions clearly are different in erythromyeloblastic leukosis from those involving the vaccinia virus (*12*), for example. In leukosis, the enzyme is not present in normal plasma but appears only in proportion to the number of particles with inception of the disease, and at no time does it exist in a state in which it can be separated from the particles by electrophoresis or sedimentation. On the contrary it must be assumed that the enzyme is either an integral part of the particle or that adsorption of the enzyme must have occurred while the particle was inside the cell.

The full significance of the variation seen in the observed interrelationships cannot be assessed at the present. Certainly some of the variation must have been referable to the methods employed, such as factors influencing the precision of the procedures for counting the particles, for estimating enzyme activity, and for titration of virus infectivity. It can be said that the precision of the different methods applied to repeated studies on a given plasma or dilution of the plasma was far greater than the variations observed from one plasma to another. It is evident that although factors were known which would influence the different determinations (*5*), much of the observed variation must have been due to biological differences in the virus particles from one host to another. The state of activity of the individual particles and the total population in a given host can hardly be static any more than that of bacteria in a given culture. Instead, the maturity of the particle, its age, the biological influence of the host on the particle as well as physical and chemical factors such as temperature, *p*H, salt concentration, and distribution in the plasma environment beyond the natural intracellular habitat of the virus might all bring about changes in the particles to influence infectivity and enzyme activity. In this report it was not possible to choose donors all in exactly the same state of the disease. Further than this, it is not possible to assume that all particles possess the same qualities in like amount at the moment of their origin.

Little work analogous to that described here has been carried out with other viruses. A brief comparison can be made with some results of studies with bacteriophage (*13*). Here, despite the relatively ideal conditions of controlled virus growth in standard media; of an excellent method for counting virus particles; and for the measurement of virus activity in the ideal host, there was observed a more than 3-fold variation of 0.4 to 1.4 in the ratio of phage particle count to infectivity. In the work with leukosis virus the maximum variation in this ratio was approximately 10-fold with the 31 plasmas and about 7-fold with 27 of the total number. If the character and complexity of the problem with leukosis are considered, the variations observed in the present work do not seem beyond the

limits of reasonable biological differences. In this respect the results are indicative not only of the existence of interrelationship between particles, enzyme and infectivity but constitute a description of the magnitude and character of the variation to be expected.

The results of the present study of plasma infectivity corroborate in many respects the data previously reported (*14*) indicating a wide variation in the infectivity of plasmas from different hosts with erythromyeloblastic leukosis. It should be emphasized, however, that the conditions of the earlier work were entirely different from those of the present studies. In the former case the donors used were chosen from a large population of diseased chicks on the basis only of 1) the length of the interval after inoculation of the bird and 2) the presence of a high primitive-cell count. At that time the latter was the sole immediate criterion of the probable state of the disease in the chick. In the present work, preliminary selection was made also on the basis of the primitive-cell content of the blood, which could be estimated approximately from the routine blood smears, but the final choice was based on the capacity of the blood to dephosphorylate adenosine triphosphate in the micro screening test. It seemed desirable to employ the youngest donors but some older ones were taken either because young birds were not available at the time or because of other factors, such as high enzyme activity (O 354, table 1) in the older donors. Further, in every experiment an effort was made to select a group of donors of greatly different individual activities within the limits of approximately 5, 10, 15, or less than 5 minutes reaction time in the micro test. As a consequence, about 50 percent of the birds were discarded immediately (despite a possibly high primitive-cell content) as undesirable for study (reaction time greater than 15 minutes). This eliminated at once not only any elements of random sampling but also practically all of the chicks which might have shown noninfectious plasmas like those seen in the earlier work. A further selective influence was the loss by death of many of the chicks of shortest reaction times between the screening test and bleeding for plasmas the following day. Because of these influences it is likely that the donors used were representative of scarcely more than a third of the total population. This provides a ready explanation of the smaller variation in individual infectious capacities of the plasmas, less than 1.5 $\log_{10}$ intervals of dilution (except in one case, text-fig. 1), than the more than 5-dilution variation seen in the earlier work. Only one noninfectious plasma was encountered in this study.

Another considerable difference between the two sets of results is the apparent levels and extent of variation of particle counts. In the earlier work the range reported was 0.1×10^9 to 7.2×10^9 particles per ml. plasma. The lowest in the present work was 6.6×10^9, but the range extended upward to $1{,}754 \times 10^9$, a 250-fold range as compared with the previous one of 72-fold. The higher level in the present work was due in part, undoubtedly, to the screening test which made possible the deliberate choice of the most highly active plasmas. It is probable, also,

that much of the difference was due to known improvements in the technique of counting the particles, which obviously would tend toward the observation of higher counts. This would account for some of the discrepancy between the former and the present work. In the earlier studies no correlation was observed between particle count and infectivity. Continual experience has led to progressive refinement in the over-all sequence of procedures (*5*) with the result that relations obscure in the beginning become evident with better understanding and control of the conditions of the experiments.

The question will be raised in future work of the relation of the primitive cells in the circulating blood to the presence of the virus in the blood. It seems likely that the virus is present in these cells, evidence for which has been obtained in preliminary work, and that the agent either escapes from the infected cell or is liberated with breakdown of the primitive cells. Nevertheless, it has been clear from the present work and results reported elsewhere (*8*) that the virus and the enzyme activity of the blood are by no means quantitatively related to the number of primitive cells circulating at a given time. The finding indicates that a high concentration of primitive cells is not necessarily pathognomonic of high virus activity but that the latter does not occur in the absence of a high cell count.

Summary

Studies were made on the relation of a specific particulate component to the infectivity of plasmas from chicks with erythromyeloblastic leukosis. A definite correlation between the particles and infectivity was found: 1) in the plasmas with onset of the disease in chicks inoculated with the virus; 2) in the electrophoretic mobilities of the particles and the infectious unit; and 3) in the sedimentation properties of the two materials of the plasma. These findings are interpreted as evidence that the specific particles constitute the virus of the disease. Work carried along simultaneously with the present investigations has revealed a close relationship of the same particles to an enzyme in the plasma acting to dephosphorylate adenosine triphosphate. The results of all these studies provide a sound basis for regarding the particles as the virus which possesses the specific enzyme activity.

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Dose-Response Relations in Experimental Transmission of Avian Erythromyeloblastic Leukosis. III. Titration of the Virus [1, 2]

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In an earlier report (*1*) there were described certain of the characters of host-response of 3-day-old chicks to the virus of erythromyeloblastic leukosis administered experimentally. Following inoculation of virus, usually in the plasma from a bird with the disease, there was observed an essentially linear relation between log dose and the reciprocal of the latent period, and the same between log dose and the incidence of positive inoculations expressed in probits. The relations were such that either response could be employed with practical utility for bioassay or titration of virus infectivity. However, principally for reasons based on the lack of a satisfactory means for weighting the individual observations of latent period, the relation concerned with incidence expressed in probits was used in analyzing the results of routine virus titration. Several deficiencies in this procedure, too, were evident: 1) the slope of the regression of incidence on dose was extremely shallow, which limited the accuracy of the assay; 2) there was wide variation in the slope; and 3) there occurred an occasional deviation of the relation from linearity. A further serious impediment to large-scale work was the relatively high average resistance of the test hosts then in use, New Hampshire Red chicks, to infection with the virus as well as extreme variation in individual resistance or susceptibility.

Following the work reported earlier, many studies have been made of host-response to the virus in routine titrations. With the advantage of the large number of data available, there is now a better understanding of the real nature of the interaction of the chick host with the agent. It has been recognized that although the time-frequency of positive responses to a given dose is normally distributed in the initial phase of the response, the subsequent portion of the distribution deviates markedly, except in special instances, from the normal. Statistical procedures are available for the analysis of such incomplete or truncated distributions, and these have been applied to the present problem. The analyses, making use of the trend

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[3] Scholar in Cancer Research of the American Cancer Society, Inc.

of those data within the normal distribution, have revealed a closely linear relation between log dose and the median log latent period. In addition there has been found a special strain of test chicks of unusually high susceptibility to infection with the virus. The application of the statistical procedures to data obtained with the special chick hosts has given results of relatively high accuracy. The findings are described in the present report.

Materials and Methods

The studies were made on a series of 29 plasmas from as many individual chicks, which were the same ones [table 1 of (*2*), except O 935] used in studies (*2*) of the interrelationships of particle count, enzyme activity, and infectivity. The origin, method of preparation, and character of the plasmas have already been described (*2*) in detail. The manner of preparing and injecting the inoculums as well as of making the diagnostic blood smears was likewise described in the same report. Experiments were made also on 8 separate pools of plasmas which are listed in table 1. Plasma for the pools was obtained and treated as were the individual plasmas with a single exception. After the cells were removed by centrifugation, the plasmas were mixed to form the pools, and these were filtered *without* prior dilution. Dilutions were then made, as shown in table 1, with BSA-Simms' solution (*1*), and injections were carried out as before (*1*, *2*) in the number of chicks indicated also in table 1.

TABLE 1.—*Results in the titration of the infectivity of the erythromyeloblastic leukosis virus in eight plasma pools*

Exper. No.	Number of plasmas	Log dose plasma (*ml.*)	Number of chicks inoculated	Corrected number of chicks inoculated	Median log latent period (*days*)	Total percent positive	Percent positive at truncation*
59	11	−3	90	84	1. 273	98. 8	81. 0
		−2	90	81	1. 154	100	100
		−1	90	86	1. 101	100	100
60	5	−3	80	59	1. 311	100	91. 3
		−2	80	68	1. 173	100	100
		−1	80	69	1. 056	100	100
72	12	−3. 897	90	78	1. 431	75	67. 9
		−2. 497	90	78	1. 288	97	87. 9
		−1. 097	90	69	1. 134	100	100
73	11	−3. 398	60	38	1. 308	94. 7	84. 2
		−2. 398	60	47	1. 227	97. 9	93. 6
		−1. 398	60	50	1. 116	98	94
74	13	−3. 398	60	26	1. 351	88. 5	80. 8
		−2. 398	60	30	1. 245	93. 3	70
		−1. 398	60	25	1. 104	100	100
75	13	−3. 319	55	39	1. 303	92. 3	84. 6
		−2. 319	55	40	1. 217	100	92. 5
		−1. 319	55	30	1. 104	100	100
76	12	−3. 273	45	29	1. 334	75. 9	51. 7
		−2. 273	45	22	1. 240	95. 5	72. 7
		−1. 273	46	27	1. 123	100	100
77	12	−3	60	50	1. 272	94	92
		−2	60	48	1. 163	95. 8	93. 7
		−1	60	48	1. 066	97. 9	97. 9

*See text and text-figure 1.

The test chicks serving as donors of the plasmas containing virus and as the host population for titration of virus infectivity were White Leghorns obtained from the Regional Poultry Research Laboratory, Agricultural Research Administration, U. S. Department of Agriculture, East Lansing, Michigan. These birds were of the inbred line 15 (*3*, *4*) developed in that laboratory since 1939. The strain has been inbred for high susceptibility to *lymphomatosis*. Attention was drawn to the potential value of the strain in studies on erythromyeloblastic leukosis in an investigation, about 2 years ago, on a comparison of the susceptibilities of chicks from various sources to infection with the viral agent of this disease. The results of studies on several lines of White Leghorns from the East Lansing Laboratory as well as on birds from other sources will be reported in another paper (*5*). In July 1952, about 600 hens and pullets of line 15 were obtained from the Regional Poultry Research Laboratory and housed in an adequate laying house adjoining the laboratory at Duke. All the chicks of the present work, except those of experiments 59 and 60, were the offspring of this original group of layers; eggs for experiments 59 and 60 were shipped to Duke from layers at East Lansing. The eggs were collected twice daily and held at about 65 to 70° F. A batch of eggs was put into the incubator each Saturday morning so that the hatch came off on Sunday and Monday. The baby chicks were placed on litter on the floor under a good brooder and then separated into the appropriate titration groups and marked with dyes of various colors on Tuesday afternoon. Injections were made routinely on Wednesday morning, so that, with respect to the hours of incubation and the time after hatching, there was a variation of not more than 2 to 4 hours from one weekly batch to another. The injected chicks were put into another pen on the floor and kept there until the following Wednesday morning at which time wing bands were inserted, and the chicks were placed on wire in battery brooders. Those surviving the stage needing special brooder-heat were kept on wire in standard broiler batteries.

As chickens, the birds were relatively poor specimens. The productivity of the hens was low, about 114 eggs per hen per year; the fertility of the eggs only about 69 percent; and the hatchability of this number of fertile eggs was 73.6 percent. This was based on the records on 583 hens laying 35,430 eggs over a period of 195 days. The baby chicks were small and of only moderate vigor. They were fed a mixture of equal parts of very finely cracked corn and wheat for 2 days after hatching, and this was followed by a standard baby-chick starting mash. It was most important to maintain the relative humidity of the chick room at levels above 50. Furthermore, it was necessary to keep water and feed, especially the former, constantly available to the chicks until a few minutes before injection and to keep the birds warm. After the injection into the wing vein, as already described (*1*, *2*), the individual groups were kept quiet in a baby-chick crate until the danger of further bleeding from the vein puncture had passed, about 20 minutes, and returned promptly to water and feed. Without these precautions, the immediate loss after injection was very

serious. It was sometimes necessary to use chicks from pullet eggs, and these were especially vulnerable to adverse influences. The extent of the loss before the importance of the various factors was realized is revealed in table 1 in a comparison of the number of chicks inoculated and the corrected total inoculated. Most of the deaths occurred during the first week of life both before and after injection. It is notable, however, that the loss of inoculated birds did not affect the characters of the final response other than to diminish the number of birds available for analysis. This was clearly shown by the similarity of the results obtained when the death rate was high, in the early experiments, to those observed when better conditions had been established.

Results

Table 2 gives the summary of a typical response of line 15 chickens to inoculation with graded doses of filtered leukotic plasma (experiment 77, table 1). The three dose-groups of chickens received successive tenfold dilutions of plasma. Each group initially contained 60 chicks. Because of the early deaths of some of the birds, correction was made (*1*) for the total number available for analysis. The three righthand columns of table 2 record the number of chickens becoming ill with the disease on each date after inoculation as shown by blood smears.

Two important characteristics of the response are to be noted: 1) the latent periods of the individual chickens in a dose-group were spread over relatively short periods of time (5 days for dose 10^{-1} ml. of plasma to 11 days for dose 10^{-3} ml.); and 2) the increase in the latent periods was relatively sharp as the dose was decreased. Thus, all of the susceptible

TABLE 2.—*The results of experiment 77*

Latent period		Dose log ml. plasma		
Days	Log days	—3	—2	—1
10	1. 000			3
11	1. 041			15
13	1. 114		8	23
14	1. 146		9	5
15	1. 176		10	1
16	1. 204	3	15	
17	1. 230	9	3	
18	1. 255	10		
20	1. 301	17		
21	1. 322	4	1	
22	1. 342	1		
23	1. 362	2		
27	1. 431	1		
Total positive....		47	46	47
Corrected total inoculated.....		50	48	48
Percent positive..		94. 0	95. 8	97. 9

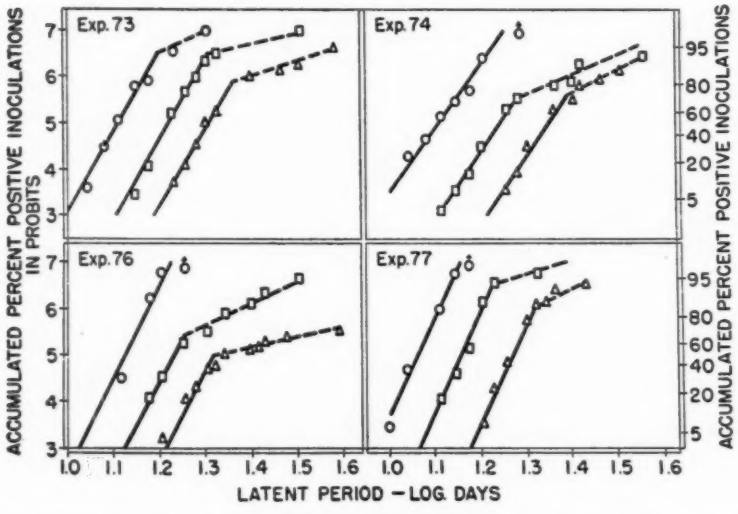
chickens receiving the 10^{-1} ml. dose had developed the disease before the first chicken receiving the 10^{-3} ml. dose became positive. This is in contrast with the response of the New Hampshire Red chicks reported previously [(*1*), table 1] where overlapping was considerable over even a broader range of doses. Another feature of the response of line 15 chickens, as shown in table 2, is that practically all chicks were susceptible to all three doses, another very great difference from the response of New Hampshire Reds. In experiments with plasmas of lower infectivity, this, of course, was not true, but it was common for all chicks to be susceptible to 0.1 ml. of the specimens of filtered plasma employed in the present experiments.

Repeated observations, such as those of table 2, emphasized the superiority of the White Leghorns over the New Hampshire Red chicks as the test host in titration of the virus. Because of the known imperfections in the previous analyses based on the reciprocal of latent period or on incidence, other relations of greater promise were sought. The high susceptibility of the chicks ruled out the use of the quantal response except under special conditions difficult to reproduce from one experiment to another. The characters of the time relation seen in table 2 and in similar arrays of other data suggested further search for latent period relations more ideally applicable to the problem than the one used earlier. It was important to find some reproducible relationship for describing the response of the individual chicken to the agent. The desirable result, both from the point of view of statistical analysis and the biological interpretation of the response of the chicken population, would be to fit the response or some function of it to the normal distribution curve. This would make available a technique for expressing the average response of the chicken population; the variability of the responses of the individual chickens about this average value; and the error of these estimates. It was obvious that latent period, expressed in days, would not follow such a distribution. Several transformations of the data were tried, and, as will be seen, certain aspects of the latent period expressed as log days gave a good fit to the normal distribution curve.

Text-figure 1 presents an analysis of the host-response data in terms of time-frequency relations. These are the results of the inoculation of four different filtered plasma pools, experiments 73, 74, 76 and 77 (table 1), into 3-day-old chicks from separate hatches of the line 15 chickens. The time axis (abscissa) is expressed in terms of log days after inoculation. The response is given as the accumulated percentage of positive chickens in each dose-group plotted on the probit scale (*6*), and each point represents the accumulated percentage positive on a given day. Inspection of the points revealed an apparently linear or normal distribution of some of the data, for example, those obtained with the largest doses of plasma in experiments 74, 76, and 77. In all other cases the data seemed to follow a normal distribution through a part of the range and finally to deviate sharply from it. The characters of this truncated (*7*) distribution are better shown by the lines drawn through the points by inspection. In a

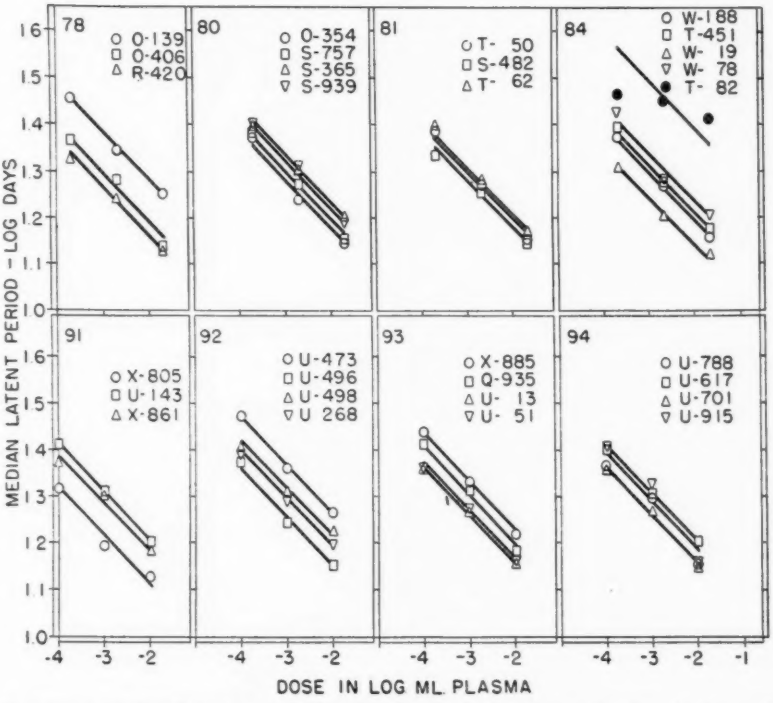
given experiment the slopes of the lines for all dose groups in the region of apparently normal distribution did not differ significantly from one another, so that in practice a common slope could be selected which would best fit all of the points in this region in the experiment. The straight lines through the truncated portions of the distribution in the various dose groups of the different experiments were drawn by inspection as the best apparent fits to the data. The distinctive features of the truncation are strikingly illustrated, for example, by the results of experiment 76 in text-figure 1. The points obtained with the highest dose follow the line of normal distribution to 100 percent positive inoculations, only to 64 percent positive with the next lower dose, and to 50 percent positive with the lowest. The data as plotted indicate that the response of chicks to different doses was similar through a certain time interval, except that the median latent period increased as the dose decreased. However, when this interval of observation was extended sufficiently, the response of the host was modified, and a new distribution of susceptibility prevailed. After this interval, at the truncation point, there was a decrease in the rate at which chicks became positive. It is to be noted that the truncation was not related to the total period after inoculation or to a given percentage positive, but was dependent on the dose.

In the preceding discussion it was assumed that the data in the *first part of the truncated distribution* followed the normal distribution curve. Statistical analyses have not been made to determine whether or not this is actually the case. Nevertheless, simple inspection of the data in


Exp. 73
Exp. 74
Exp. 76
Exp. 77
ACCUMULATED PERCENT POSITIVE INOCULATIONS IN PROBITS
ACCUMULATED PERCENT POSITIVE INOCULATIONS
LATENT PERIOD — LOG. DAYS
1.0 1.1 1.2 1.3 1.4 1.5 1.6
3 4 5 6 7
95 80 60 40 20 5

TEXT-FIGURE 1.—Time-frequency distributions of chick host-responses to graded doses of the virus of erythromyeloblastic leukosis in four separate experiments with different plasma pools from diseased chicks. The points with arrows upward signify 100 percent responses.

numerous experiments such as those of text-figure 1 shows that the fit of the individual points to the straight line expected with normal distribution is exceedingly close. The results of practical application of the analyses based on validity of the assumption justify use of the analytical procedure in this region of the distribution in the estimation of virus infectivity. This is illustrated in text-figure 2 in the findings in the titration of a series of 29 plasmas in 8 separate experiments. In each of these experiments the infectivities of 3 to 5 plasmas from individual donors were measured. The median log latent periods were determined by the above method for the three doses of each plasma spaced at 10-fold dilution intervals, and were plotted against dose in log ml. of filtered plasma.

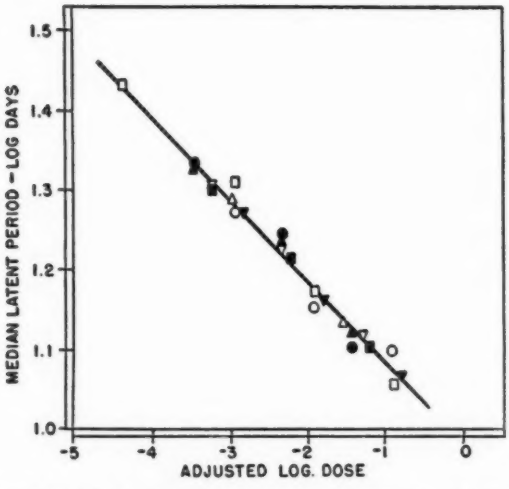


TEXT-FIGURE 2.—Relation of median log latent period in days to log dose in the results of the titration of the virus in 29 different plasmas from individual chicks. These are the same plasmas described in table 1 of (*2*).

Regression lines were calculated for each plasma by the method of least squares, weighting the points as suggested by the method of Bliss for truncated normal distributions (*7*). The uniformity of the slopes was such that a common regression line could be calculated and applied to all experiments. The slopes for all of the individual plasmas in the 8 experiments varied from 0.092 to 0.117 (exclusive of T 82, experiment 84)

with a common slope of 0.104. All of the lines shown in text-figure 2 were drawn with this common slope through the means determined for each plasma. The fit of the points to the lines is excellent. The one plasma of low potency in experiment 84, text-figure 2 [T 82, table 1 of (*2*)] gave a poor fit which may indicate that the limit of the method was approached.

In text-figure 3 is shown the similar relation for the results with the eight plasma pools, table 1. The points were adjusted along the dose axis to a common potency so as to bring the mean of each experiment to the common regression line. The fit of the points to the line is again excellent. The common slope, 0.104, is identical with that calculated from the data obtained with the individual plasmas.



TEXT-FIGURE 3.—Relation of median log latent period in days to adjusted log dose in the results of the titration of the virus in eight different pools of plasma from diseased chicks (table 1). The individual points lie about the line drawn through the common mean response and dose of all of the experiments. The slope of the line is the average slope for the results of the group.

The method for measuring host susceptibility may be compared with that previously presented (*1*) based on the percent positive inoculations at each successive dose level. Most striking is the greater precision of the dose-response relations when median log latent period is used in the manner described as the basis for comparison. An analysis of the quantal responses observed in New Hampshire Red chicks as previously reported [table 4 of (*1*)] gave values of 2.6 to 4.5 for λ (*8*, *9*), whereas the value of λ was 0.6 for the present latent period method using line 15 White Leghorn chicks. It can be seen, then, that the median log latent period derived by application of the procedure for truncated distributions, gives a more direct and accurate measure of the susceptibility of the population to the virus or, on the other hand, of the degree of virus infectivity.

Discussion

The results of the present work have revealed relationships between median log latent period and log dose yielding estimates of virus infectivity with considerable accuracy in the titration of the virus of erythromyeloblastic leukosis. In the experiments described here and in others, using 250 to 78 (corrected total inoculated) chicks for each sample titrated, the average standard deviation, determined from the observed variations of the individual points about the respective regression lines, was ± 0.13 log ml. plasma. Thus in 2 of 3 measurements, the resulting values would be expected to lie within about ± 35 percent of the true value. A notable feature in the analyses was the remarkable uniformity in the slopes of the regression of median log latent period on log dose. That the relation is linear, within the limits of the experiments, is highly probable as seen in text-figures 2 and 3. It should be emphasized that this uniformity has prevailed in the present experiments in all of which the test host was the special strain of White Leghorn chicks. The small variation in slope is valuable in view of the possibilities in extensive titration work. If sufficient data confirm the present indications, it is likely that the slope, at least for these chicks, may be regarded as a constant and that more accurate estimates may be obtained by fitting data to this slope rather than to the various slopes calculated in individual titrations.

Another important factor contributing to the accuracy and utility of these analyses is concerned with the homogeneity of the test-chick population with respect to susceptibility or resistance to infection with the virus. This is particularly well illustrated in text-figure 1 in which it is seen that the slopes of the individual first limbs of the truncated distributions are approximately the same throughout the series of four titrations. Calculations with all of these data of the present report, as well as those of other experiments, confirm the appearance of uniformity of the slope and give the value ± 0.057 log days as the standard deviation of the distribution of individual log latent periods. Judging from these observations, it would appear that the state of susceptibility or resistance of the White Leghorn chicks through this portion of the response is likewise essentially a constant.

It is obvious from the foregoing that many of the advantages gained in the application of the median log latent period–log dose analysis are derived through use of the special strain of White Leghorn chicks. In addition to the statistical considerations, there are other factors which bear on the labor, time and cost of titration. A comparison of data with New Hampshire Red chicks with those obtained with the White Leghorns is made in this respect in table 3. The data were observed in two experiments, experiment 31 with New Hampshire Reds and experiment 92 with White Leghorns, in each of which 4 individual plasmas were titrated in 3 dilutions each. It is seen that the number of White Leghorn chicks used was far less than that of the Reds though the total number of White Leghorns diseased was much the greater. These diseased chicks quickly made their contribution to the titration data and then were killed to provide plasma for other experiments or died early of the disease. This

not only lessened the expense of feeding but also the number of blood smears. Statistically, the usefulness of the White Leghorn chicks was much greater than indicated in table 3. On the basis of a constant standard error of a log potency estimate, the accuracy expected with a given number of White Leghorns and the median log latent period response would be from about 4.3 to 7.5 times greater than that expected with twice the number of New Hampshire Red chicks and the quantal response employed previously. In practice it has been convenient to maintain the number of White Leghorns used at the level previously established with the New Hampshire Reds. As a consequence, the number of experiments has been doubled and the expense of care and feeding halved, despite which the accuracy of the log potency estimates has been increased 4- to 7-fold. The principal disadvantages are the need for maintaining a laying flock and the complications associated with incubation of the eggs. Practically, however, these difficulties are incomparably overshadowed by the advantages.

TABLE 3.—*Comparison of New Hampshire Red with White Leghorn chicks (line 15) in the numbers employed for titration of 4 plasma specimens and in the factors influencing the cost and time of the experiments*

	New Hampshire Red chicks (*Exper. 31*)	White Leghorn chicks—line 15 (*Exper. 92*)
Total inoculated	720	480
Total infected	194	365
Total living at end of experiment	520	70
Smears	5817	4125
Period of experiment (*days*)	57	43
Average life of diseased chicks (*days*)	8. 8	5. 4
Average life after inoculation (*days*)	40. 6	26

The phenomenon of truncation of the time-frequency distribution has been interpreted as associated with a change in the level of host susceptibility due to the induction of acquired immunity in a portion of the respective dose groups during the onset of the infectious process. Truncation, though of a somewhat different type, has been observed also in the response of rabbits to the papilloma virus, the implications of which have been discussed in detail (*10*). It is probable that the differences between the response of the rabbits, which shows a double truncation, and that of the chicks are dependent on the different conditions of the experiments. Each rabbit was inoculated with multiple graded doses of papilloma virus so that the larger doses could affect the course of infection with the smaller doses in the same animal. In contrast each chick received a single dose of virus which was responsible for stimulation of both the infectious process and the state of acquired immunity. Thus, the point of truncation was determined by the time available for the induction of immunity in the individual chicks and this in turn was dependent on both individual susceptibility and the dose employed. After this period, all of the remaining chicks of the dose group were affected in a related manner, giving rise to

the single truncation. If the dose was sufficiently large, infection came too quickly to permit induction of the immune process; the response was related to the natural state of resistance; and truncation did not occur.

It has been observed (*11*) previously that the chick host-response to inocula containing washed primitive cells exhibits the same characters as the analogous response to the virus of erythromyeloblastic leukosis. Analysis of experiments reported earlier has shown also the same type of truncated distribution. The occurrence of the phenomenon in the measurement of transmissibility of the disease by means of cells seems to provide some explanation of the deviation from the linear relation of incidence expressed in probits to log dose seen in those studies. The deviations occurred either with very large doses of cells, which were associated with high incidence and short latent periods, or with the smallest doses. In the first instance, the acquired immunological influences made little or no impression on response, while in the second acquired immunity far outweighed the natural state of resistance. In the region between, the influences of natural and acquired resistance were more nearly balanced, resulting in a response related to both states.

Summary

Further studies have been made of the host-response of 3-day-old chicks to experimental inoculation with the virus of erythromyeloblastic leukosis. It was observed that, except under special conditions, the time-frequency relations for a given dose were truncated, consisting of an initial limb of normal distribution followed by a sharp deviation. The point of truncation was related directly to the dose. A remarkable uniformity was seen in the slopes of the first limbs of the distribution, not only with different doses in a single experiment but also in the various doses from one experiment to another. Analyses showed that the median log latent period of the chicks represented in the limb of normal distribution was related in a linear fashion to log dose and that this relation provided an accurate practical means for the estimation of either virus infectivity or the state of resistance or susceptibility of the test-host population.

There has been found a special line of White Leghorn chicks which are of relatively very high susceptibility and homogeneity to infection with the virus. The origin of the chicks and the characters of their superiority over other chicks are described.

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Dose-Response Relations in Experimental Transmission of Avian Erythromyeloblastic Leukosis. IV. Strain Differences in Host-Response to the Virus [1, 2]

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There have been developed (*1–3*) at the Regional Poultry Research Laboratory, East Lansing, Michigan, several lines of White Leghorn chickens inbred with respect to resistance or susceptibility to *lymphomatosis*, the lymphoid type of the leukosis complex (*4*). During the years intervening since the beginning of the breeding program in 1939, definite differences have been observed between the responses of the respective lines to exposure to the factors responsible for the disease under natural conditions. In 1952 it seemed desirable to determine whether resistance or susceptibility to lymphomatosis might be reflected in the response of these lines to experimental inoculation with the virus of *erythromyeloblastic leukosis*, which is the leukemic form of the leukosis complex. It was rather surprising to learn that no systematic relation could be demonstrated and, further, that some of the lines exhibited relatively very high susceptibility to the agent of erythromyeloblastosis. These results were of interest, not only in their bearing on possible relations between lymphomatosis and erythromyeloblastic leukosis, but also in the finding of a host population so much more useful in the study of erythromyeloblastosis than the New Hampshire [4] chicks employed (*5*, *6*) until that time. Because of the possibility that White Leghorns from other sources might display a like high susceptibility to the virus, further studies were made for comparison of the respective levels of responses of New Hampshire chicks; chicks of several different lines of White Leghorns from the Regional Poultry Research Laboratory; and White Leghorns from two other sources. The results of this work, carried out in collaboration between the laboratories at East Lansing and Duke, are described in the present report.

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[2] This work was supported by a grant to Duke University from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service; by a grant from the American Cancer Society, on recommendation of the Committee on Growth; and by the Dorothy Beard Research Fund.

[3] Scholar in Cancer Research of the American Cancer Society, Inc.

[4] The designation "New Hampshire" chicks in this report is synonymous with the terminology "New Hampshire Red" chicks employed in other publications from this laboratory.

Materials and Methods

New Hampshire chicks.—At the time, 1949, of the initiation of the work on erythromyeloblastic leukosis at Duke (*5*), there was no experimental basis for the selection of a particular breed or strain of chickens for use in the titration of the etiological agent. It had been recognized earlier (*7*) that 3-day-old chicks were much superior to older birds in their susceptibility to the virus. Since New Hampshire chicks could be obtained regularly through the cooperation of a large local hatchery, these birds were chosen for routine studies. Preliminary experiments failed to reveal significant differences in the responses of chicks from different flocks of New Hampshire laying hens. However, in order to preserve, as well as possible, uniformity in the population to be used over a long period of time, chicks were obtained always from the eggs of three large flocks which were of common derivation. All three of these groups of layers were of the "Nichols" strain recently procured from the flocks at the Nichols Breeding Farm, Kingston, New Hampshire. The birds were brought to the laboratory at 1 day of age, kept on wire in battery brooders, and inoculated with virus at 3 days of age. They were relatively large, vigorous individuals and presented few problems in the brooding and growing stages.

White Leghorns from the Regional Poultry Research Laboratory.—Studies were made on chicks of 6 different inbred lines of White Leghorns from this source. Eggs were gathered from layers of the various lines at East Lansing and shipped to Duke by air at intervals of 2 weeks. Special care was taken to keep the eggs of the different lines separate. Incubation was carried out by a responsible hatchery man [5] under the conditions essential for keeping the groups of chicks apart. The chicks were delivered to the laboratory the day after hatching and marked immediately with different dyes for identification of the various groups. No other White Leghorns were present in the laboratory at that time. Inoculations with virus were made as usual on the third day of age. The chicks were very small, and hatched slowly and with difficulty. Losses were numerous in the first week after hatching as described elsewhere (*6*).

Farm Bureau White Leghorns.—A batch of White Leghorn chicks was obtained from the Pennsylvania Farm Bureau Hatchery, Harrisburg, Pennsylvania. These birds were healthy, vigorous specimens.

Dembro White Leghorns.—These chicks were purchased from the Dembro Poultry Farm, Sewell, New Jersey, and were shipped by train immediately after hatching. The birds were unusually large and vigorous.

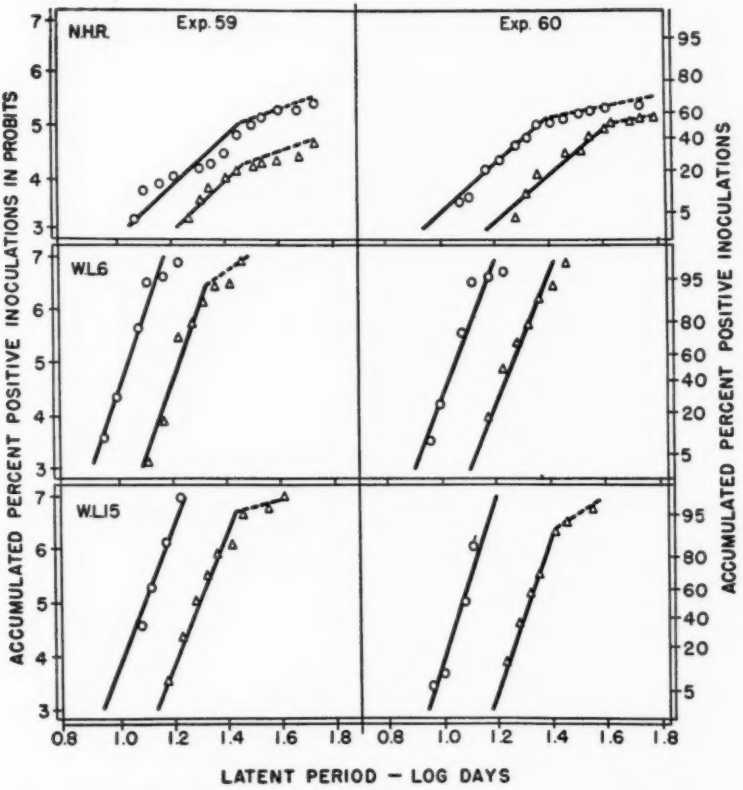
The techniques of virus-inoculation and diagnosis of onset of the disease were those routinely practiced here (*5*, *6*).

Experiments and Results

Experiments 59 and 60.—These were duplicate experiments made 2 weeks apart. In each, the response of New Hampshire chicks was compared, as control, with that of White Leghorns of the Regional Poultry

[5] The authors wish to express their appreciation of the generous cooperation of Mr. Lacy Thomas in this aspect of the work.

Research Laboratory lines 6 and 15. A pool of plasma was obtained from diseased New Hampshire chicks, which, after removal of the cells, was filtered through Selas candles (*6*). In both experiments each of the three batches of chicks was divided into three groups, and these were given doses, respectively, of 10^{-1}, 10^{-2} and 10^{-3} ml. of plasma. The numbers of chicks in each dose group are given in table 1. The results of the two experiments are summarized in table 1 and illustrated graphically in text-figure 1.



TEXT-FIGURE 1.—Time-frequency distributions of the responses, in duplicate experiments (experiments 59 and 60), of New Hampshire (N. H. R.) and White Leghorn chicks of inbred lines 6 and 15 (W. L. 6 and W. L. 15) to doses of 10^{-1} and 10^{-3} ml. plasma (table 1).

Experiments 61 and 62.—In these two similar experiments, made with an interval of 2 weeks between, there were compared the responses of New Hampshire chicks with those of chicks from the Regional Poultry Research Laboratory—White Leghorns of lines 7, 9, 10 and 14. As in experiments 59 and 60, the plasma was obtained in a pool from diseased New Hampshire chicks. The plasma received the same treatment as

TABLE 1.—*Results of experiments 59 and 60*

Exper. No.	Host chicks	Log dose plasma (*ml.*)	Corrected No. chicks inoculated	Median log latent period (*days*)	Standard deviation*	Total percent positive	Percent positive at truncation	Log latent period at truncation (*days*)
59.....	New Hampshire.....	−1	79	1. 522	0. 262	64. 6	55. 7	1. 556
		−2	79	1. 627	0. 260	55. 7	48. 1	1. 644
		−3	77	1. 922	0. 228	37. 7	21. 0	1. 556
	Line 6.....	−1	89	1. 039	0. 060	100	94. 4	1. 176
	(White Leghorns)	−2	88	1. 141	0. 057	98. 9	94. 3	1. 279
		−3	100	1. 233	0. 068	100	92. 0	1. 362
	Line 15.....	−1	86	1. 103	0. 061	100	100	.....
	(White Leghorns)	−2	81	1. 152	0. 075	100	100	.....
		−3	84	1. 281	0. 077	98. 8	81. 0	1. 362
60.....	New Hampshire.....	−1	80	1. 352	0. 181	65. 0	50. 0	1. 362
		−2	80	1. 606	0. 245	58. 2	57. 0	1. 672
		−3	85	1. 596	0. 202	56. 0	51. 2	1. 634
	Line 6.....	−1	80	1. 043	0. 065	98. 7	96. 2	1. 230
	(White Leghorns)	−2	79	1. 098	0. 067	98. 7	91. 0	1. 230
		−3	82	1. 244	0. 074	100	89. 3	1. 362
	Line 15.....	−1	69	1. 061	0. 064	100	100	.....
	(White Leghorns)	−2	68	1. 172	0. 080	100	91. 8	1. 279
		−3	59	1. 315	0. 077	100	91. 3	1. 415

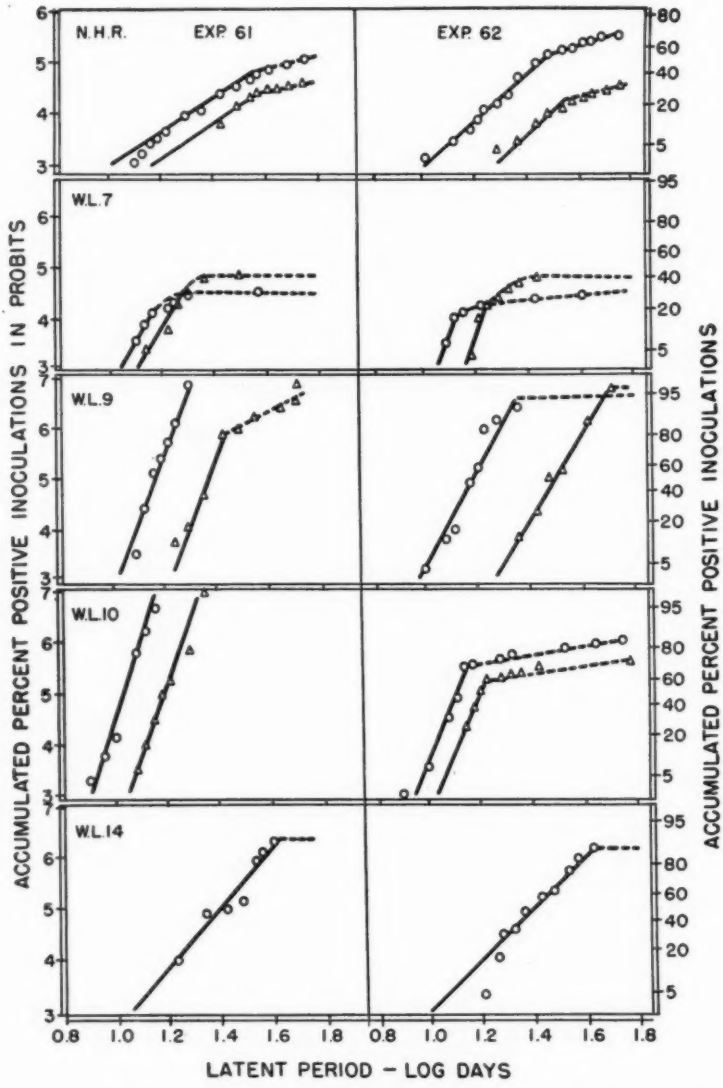
*The values of standard deviation represent the reciprocals of the slopes of the initial limbs of the truncated time-frequency distributions.

TABLE 2.—*Results of experiments 61 and 62*

Exper. No.	Host chicks	Log dose plasma (*ml.*)	Corrected No. chicks inoculated	Median log latent period* (*days*)	Standard deviation *†	Total percent positive	Percent positive at truncation	Log latent period at truncation (*days*)
61.....	New Hampshire.....	−1	81	1. 612	0. 283	52. 5	45. 0	1. 602
		−2	80	1. 647	0. 250	45. 6	34. 2	1. 556
		−3	84	1. 642	0. 163	34. 6	29. 6	1. 602
	Line 7.....	−1	37	(1. 225)	(0. 080)	32. 4	23. 0	1. 200
	(White Leghorns)	−3	38	(1. 340)	(0. 080)	44. 4	41. 0	1. 325
	Line 9.....	−1	38	1. 156	0. 073	100	100	.....
	(White Leghorns)	−3	43	1. 352	0. 077	97. 2	80. 6	1. 415
	Line 10.....	−1	48	1. 041	0. 066	100	100	.....
	(White Leghorns)	−3	47	1. 188	0. 087	100	100	.....
	Line 14.....	−1	17	1. 411	0. 141	94. 1	94. 1	1. 602
	(White Leghorns)							
62.....	New Hampshire Reds.....	−1	80	1. 432	0. 206	69. 2	48. 7	1. 431
		−2	78	1. 582	0. 209	55. 1	41. 0	1. 568
		−3	90	1. 689	0. 205	31. 1	22. 2	1. 568
	Line 7.....	−1	31	(1. 200)	(0. 100)	29. 0	20. 0	1. 150
	(White Leghorns)	−3	29	(1. 310)	(0. 100)	40. 7	31. 0	1. 255
	Line 9.....	−1	30	1. 185	0. 095	92. 0	92. 0	1. 362
	(White Leghorns).....	−3	30	1. 511	0. 121	95. 7	95. 7	1. 708
	Line 10.....	−1	52	1. 041	0. 066	100	100	.....
	(White Leghorns).....	−3	54	1. 188	0. 087	100	100	.....
	Line 14.....	−1	24	1. 407	0. 158	87. 5	87. 5	1. 613
	(White Leghorns)							

*The values in parentheses represent approximations based on data too few to provide accurate estimations.

†The values of standard deviation represent the reciprocals of the slopes of the initial limbs of the truncated time-frequency distributions.



TEXT-FIGURE 2.—Time-frequency distributions of the responses, in duplicate experiments (experiments 61 and 62) of New Hampshire (N. H. R.) and White Leghorn chicks of inbred lines 7, 9, 10 and 14 (W. L. 7, W. L. 9, W. L. 10 and W. L. 14) to doses of 10^{-1} and 10^{-3} ml. plasma (table 2). The small number of line 14 chicks permitted study with only the single dose, 10^{-1} ml. of plasma (table 2).

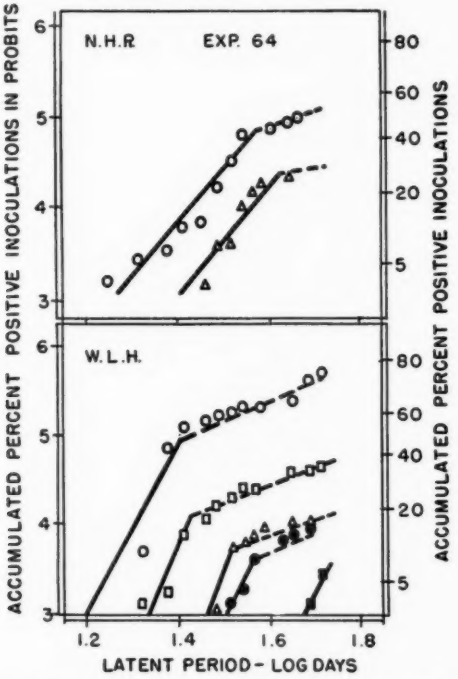
before, and the doses administered are shown in table 2. The results are also given in table 2 and in text-figure 2.

Experiment 64.—This was a comparison in a single experiment of the

TABLE 3.—*Results of experiment 64**

Host chicks	Log dose plasma (*ml.*)	Corrected No. chicks inoculated	Total percent positive	Percent positive at truncation
New Hampshire	−1	58	50. 0	44. 8
	−2	58	41. 4	37. 9
	−3	60	25. 0	23. 3
Harrisburg White Leghorns	−1	78	75. 6	52. 7
	−2	74	36. 5	13. 5
	−3	77	18. 2	10. 4
	−4	76	15. 8	7. 9
	−5	70	5. 7	.....

*The limited data do not justify calculations of the median log latent period, the standard deviation and log latent period at the point of truncation possible in the experiments of tables 1 and 2.



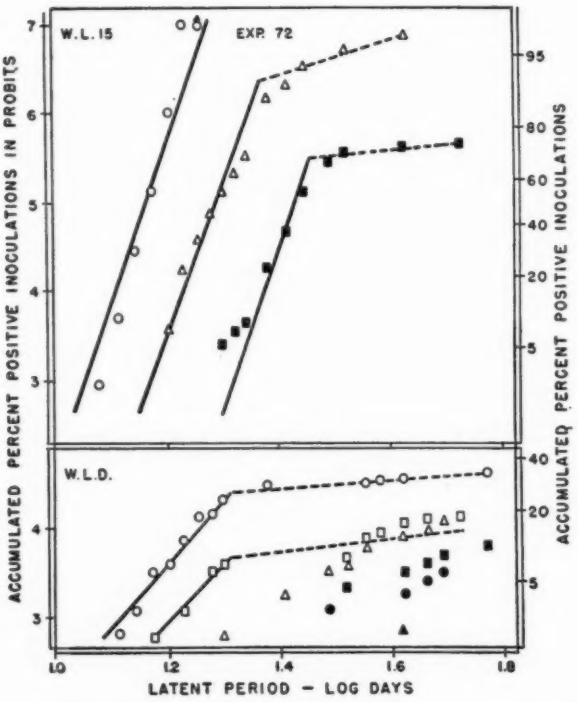
TEXT-FIGURE 3.—Time-frequency distributions of the responses, in a single experiment (experiment 64), of New Hampshire (N. H. R.) and White Leghorn chicks (W. L. H.) obtained from a commercial hatchery at Harrisburg, Pennsylvania. The responses of the New Hampshire chicks were to doses of 10^{-1} and 10^{-3} ml. plasma, and those of the White Leghorns were to doses of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} ml. plasma (table 3).

response of New Hampshire chicks with the White Leghorns obtained from the Pennsylvania Farm Bureau Hatchery. The data of this study appear in table 3 and text-figure 3. The source and treatment of plasma were the same as in the other experiments.

TABLE 4.—*Results of experiment 72**

Host chicks	Log dose plasma (*ml.*)	Corrected No. chicks inoculated	Total percent positive	Percent positive at truncation
Line 15 (White Leghorns)	−1.0	51	100	100
	−2.4	66	97.0	87.9
	−3.8	56	75.0	67.9
Dembro White Leghorns	−1.0	84	34.5	23.8
	−1.7	79	19.0	7.6
	−2.4	81	22.2	.....
	−3.1	77	6.5	.....
	−3.8	64	11.0	.....
	−4.5	67	1.5	.....

*The limited data do not justify calculations of the median log latent period, the standard deviation and log latent period at the point of truncation possible in the experiments of tables 1 and 2.



TEXT-FIGURE 4.—Time-frequency distributions of the responses, in a single experiment (experiment 72), of White Leghorn chicks of the inbred line 15 (W. L. 15) and of White Leghorn chicks from the Dembro Poultry Farm (W. L. D.). The responses of the line 15 chicks were to doses of 10^{-1}, $10^{-2.4}$ and $10^{-3.8}$ ml. plasma, and those of the Dembro chicks were to doses of 10^{-1}, $10^{-1.7}$, $10^{-2.4}$, $10^{-3.1}$, $10^{-3.8}$ and $10^{-4.5}$ ml. plasma (table 4).

Experiment 72.—This experiment was made to measure the response of chicks from the Dembro hatchery, and differed from the preceding studies in that the comparison of responses was made between those of the Dembro White Leghorn chicks and those of White Leghorn chicks derived from layers of line 15 from the Regional Poultry Research Laboratory. Prior to the time of this experiment, laying hens had been obtained from East Lansing, and a laying flock had been established at Duke. This flock and the chicks deriving from it have been described in a previous paper *(6)*. The dose-group of both kinds of White Leghorns and the inoculums of filtered pools of plasma are described, together with the results of the experiments, in table 4 and text-figure 4.

Interpretation of Results

The relation found *(6)* most satisfactory, thus far, for portraying the characters of the dose-group response of chicks to the virus of erythromyeloblastic leukosis is the time-frequency curve obtained by plotting the accumulated percent positive responses against the log latent period of onset of the disease. Basically this relation is an incomplete or truncated curve *(8)* of two limbs, examples of which are seen in text-figures 1 to 4. The first limbs of the curves have been regarded *(6)* as representing apparently linear relations, indicative of a normal distribution of responses among the individuals of the various dose-groups dependent on a normal distribution of natural resistance or susceptibility. Deviation of the curve is believed to be due to change in the resistance of some of the chicks to the virus, acquired through immunological reaction during the course of infection within the latent period. Should this be the case, then, it is only the initial limb of the curve which should depict the basic response of the chicks, and only this portion to the point of truncation is useful in interpretation of uncomplicated host-response. In some instances, the curve remains linear throughout, and this is considered as evidence that response occurs too quickly to permit interference by acquired resistance.

The significant parts of the distribution curves with respect to the present work are the slopes of the first limbs of the distributions and the location of the curves with respect to time, *i.e.*, latent period of the responses. The values of the slopes, which can be determined graphically by the method of Bliss *(8)* or calculated as suggested by Ipsen *(9)*, constitute a measure of the dispersion of the responses of individual chicks within a dose group, since the reciprocal of the slope represents the standard deviation of the responses. It has been shown *(6)* that the latent period of the onset of the disease after inoculation with the virus is related to dose and, in particular, it has been found that the relations of median log latent period, found graphically *(8)* or calculated *(9)* from the responses occurring in the first limb of the truncated distribution, are linear with log dose. This relationship has been made the basis for bioassay of virus infectivity or, conversely, of the state of host susceptibility and resistance.

It is evident from the data that variations in the responses of the different kinds of chicks occurred in several ways. No standard or control

is available for comparisons on an absolute basis. As the best substitute at the present, it would appear that the White Leghorns of line 15 approach as closely as any of the chicks to the ideal host; they are of relatively high susceptibility and, more important, have exhibited high group homogeneity (*6*) consistently, as shown by the uniformity of their responses over a long period of time. In addition much work has already been done with this line. For these reasons the results obtained are the more clearly visualized when the line 15 chicks are employed as the standard of reference in the interpretation of the behavior of the other chicks.

The responses of line 15 chicks in the present work, tables 1 and 4 and text-figures 1 and 4, were essentially identical with those previously reported (*6*). Practically all of the birds were susceptible to the doses of virus employed, and the incidence was high, also, at the point of truncation. Steepness of slope of the regressions of accumulated frequency on latent period was characteristic of the responses, indicating homogeneity of resistance within the individual dose groups. Homogeneity is evident likewise in the uniformity of the slopes of the linear distribution in repeated experiments. The other lines of White Leghorns—6 (table 1, text-fig. 1), 9 and 10 (table 2, text-fig. 2)—displayed responses essentially identical with those of line 15.

Striking differences were seen with lines 14 and 7 (table 2, text-fig. 2). The former, somewhat like line 15, was highly susceptible to virus infection, but in marked contrast with the characters of line 15 chicks, there was wide dispersion in the distribution of resistance as shown by the large standard deviation given in table 2 and seen in the spread of latent period evident in text-figure 2. A peculiar response was that of line 7 (table 2, text-figure 2). Here homogeneity was but slightly less than that of the line 15 chicks, but resistance to infection was great as indicated by the low total incidence as well as the low incidence at the point of truncation. A unique phenomenon, occurring in an identical manner in the duplicate experiments, was the greater percentage response to the 10^{-3}-ml. dose than to the hundredfold larger dose of 10^{-1} ml. plasma.

Throughout the experiments of several years, the general characters of the response of New Hampshire chicks, illustrated in tables 1, 2 and 3 and text-figures 1, 2 and 3, have been fairly uniform. The chicks are relatively resistant to virus infection, and the population is heterogeneous within the dose groups as well as in the samples of the general population employed in separate experiments made at different times (*6*). The responses are characterized by shallow slope, long latent period, low incidence and consistent truncation of the time-frequency curve. These are the factors which have contributed to the difficulties in the utilization of these birds as test hosts (*6*) for titration of the virus.

The results with the Harrisburg (table 3, text-fig. 3) and Dembro (table 4, text-fig. 4) White Leghorn chicks were not sufficient to permit calculation of slope. Inspection shows, however, that the former were somewhat more homogeneous than the New Hampshires, but they were approxi-

mately equally resistant to infection. The Dembro chicks were still less homogeneous, and the resistance of the individual chicks was the highest of all of the hosts tested. The total incidence with the largest dose was less than 40 percent, although the incidence with line 15 chicks in the same experiment was 100 percent with the same volume of plasma. Truncation was observed only with the 10^{-1} and $10^{-1.7}$ doses; with the smaller doses, it appeared that the initial limb of the curve was absent and that the responses in this region were all related to both natural and acquired resistance. This behavior was approached by the Harrisburg chicks, but in this case, truncation was seen with all doses except the smallest one.

Discussion

The present results and methods of analysis portray vividly fundamental similarities and, yet, striking differences in the reactions of various host populations of chicks to contact with the virus of erythromyeloblastic leukosis. When the results of inoculating the virus into 3-day-old chicks of different breeds, strains or lines are plotted in terms of accumulated frequency, expressed in probits, against the log latent period, there are obtained truncated or incomplete time-frequency curves. This constitutes the basic similarity which has been observed with this procedure of analysis throughout the present and earlier work (*6*). The differences between the populations investigated consist in variation in the elements or characters of the curve. Such are seen in (*a*) variation in the slope of the initial limb before truncation; (*b*) in the total incidence of positive inoculations; (*c*) in incidence at the point of truncation; and (*d*) in the median latent period determined or calculated from the characters of the responses constituting the initial limb of the curve. Interpretation of the results suggests several principal factors contributing to variation. The slope of the initial limb is determined by the homogeneity or heterogeneity of the test population with respect to the state of natural resistance. Wide dispersion of this property results in a shallow slope, while homogeneity contributes to steepness. The incidence of positive inoculations occurring before truncation appears to be related to the *natural* level of individual resistance or susceptibility to the virus, and the total incidence is a function of both natural and *acquired* resistance. Variation in median log latent period is related both to natural resistance and to the homogeneity of the population.

On the basis of these interpretations the various populations of hosts may be easily compared. It is evident that lines 6, 9, 10 and 15 exhibit relatively high homogeneity and susceptibility to the virus. Line 14 was likewise highly susceptible though very heterogeneous with respect to those factors determining natural resistance and susceptibility. In contrast, the New Hampshire chicks were both heterogeneous and resistant. Still greater resistance was shown by the Dembro chicks, but the degree of homogeneity was comparable with that of the New Hampshires and the line 14 chicks. The response of line 7 presents a larger problem of

interpretation. Obviously, individual resistance was great, though the population was relatively homogeneous. The explanation of the greater response to the smaller doses is not clear. It is possible that the effect may have been due to an immunological response different in degree or in kind from that of the other birds. In these chicks, as in all of the groups studied, the infectious and immunological processes must progress simultaneously. The point of truncation, therefore, would be dependent, in part, on the balance between the respective rates of the development of infection and the induction of acquired resistance. In birds possessing unusual capacity to respond immunologically, it is possible that the larger doses of virus might provide the stimulus for unbalance between the rates of induction of infection and acquired resistance. This, clearly, would be a response opposite that seen in the other chicks.

In the studies with the inbred lines of White Leghorn chicks from the Regional Poultry Research Laboratory, there was observed no evidence of a correlation between resistance or susceptibility to lymphomatosis and the characters of the responses of the chicks to infection with the virus of erythromyeloblastic leukosis. As described by Waters (*1–3*), lines 9 and 15 have exhibited high susceptibility to lymphomatosis, and both were found, likewise, to be susceptible to erythromyeloblastic leukosis. Lines 6 and 10 are least susceptible to lymphomatosis, but, in contrast, belong to the groups most highly susceptible to erythromyeloblastic leukosis. Line 7 is highly susceptible to lymphomatosis (neurolymphomatosis) but not to erythromyeloblastic leukosis, whereas line 14 is highly resistant to lymphomatosis but is, in contrast, highly susceptible to erythromyeloblastic leukosis.

It should be emphasized that the preponderance of data pertaining to lymphomatosis has been gathered (*1–3*) in studies of the different inbred lines of White Leghorns under conditions of natural exposure to the virus. For this reason, it might be supposed that the responses of these lines to lymphomatosis would not be comparable with the responses of the same lines to the virus of erythromyeloblastic leukosis which was injected intravenously. Grounds for doubt in this respect, however, have been eliminated recently in the study (*10*) of the responses of lines 15, 6, 10 and 7 to the virus of lymphomatosis derived in filtrates from the RPL 12 tumor. In these studies there was found a good correlation between visceral lymphomatosis occurring in these lines as the result of natural exposure and that induced by the virus in filtrates. From these results and the findings observed in the present work, it is obvious that no relation exists between the status of resistance or susceptibility to lymphomatosis and that to the agent of erythromyeloblastic leukosis. The data of the present work, therefore, constitute definite and unequivocal evidence that the agent responsible for erythromyeloblastic leukosis is different from that associated with visceral lymphomatosis.

Major variations have been revealed in both qualitative and quantitative responses of host populations of different sources and, in some of the instances, of known differences in genetic constitution. The characters

of the responses were not related to breed, *per se*, in a comparison of White Leghorns with New Hampshires. The effects of inbreeding in the different lines of White Leghorns can be seen in the relatively great homogeneity of the populations, with the exception of line 14. Even here, the change in objective (*2*) of inbreeding may be related to heterogeneity in this group. It scarcely needs emphasis that the variations observed are of much significance in interpretations of host-response in the study of infection with the virus of erythromyeloblastic leukosis.

Summary

Studies have been made of the host-response of 3-day-old chicks of different breeds and strains to intravenous inoculation with graded doses of the agent of erythromyeloblastic leukosis. The results were analyzed on the basis of time-frequency distributions which gave truncated curves, providing several criteria for judging the characters and estimating the levels of the responses. These were variation in (*a*) the slope of the first limb of the truncated curve, dependent on homogeneity of response; (*b*) incidence at the point of truncation, determined by the influence of natural resistance on response; (*c*) total incidence, which was influenced by the combined effects of natural and acquired resistance; and (*d*) the median latent period determined or calculated from the responses constituting the initial limb of the curve. This last variation was regarded as a function of both natural resistance and the homogeneity of the population. Chicks of a New Hampshire breed and White Leghorn chicks from two different commercial flocks were relatively resistant to infection with the virus and displayed marked heterogeneity in individual responses to the virus. The findings indicated no relation between either the qualitative or the quantitative characters of the responses and the two different breeds of chicks. The investigation included six different lines (6, 7, 9, 10, 14 and 15) of White Leghorn chicks long inbred (at the Regional Poultry Research Laboratory, East Lansing, Michigan) for resistance or susceptibility to lymphomatosis. All of the lines, except 7 (susceptible to neurolymphomatosis) were highly susceptible to the virus of erythromyeloblastic leukosis and all, except line 14, were relatively highly homogeneous in response to this agent. There was no evidence of correlation between the state of resistance or susceptibility to lymphomatosis and that to erythromyeloblastic leukosis.

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The Phenol-Oxidizing Enzyme System of Human Melanomas; Substrate Specificity and Relationship to Copper [1, 2]

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Although the "mammalian dopa-oxidase" or "mammalian tyrosinase" of the Harding-Passey and the Cloudman melanomas of mice were extensively studied during the last decade (*1–6*), there have been no recent studies on the phenol-oxidizing system of human melanomas. Melanoma occurs relatively rarely in man: the primary tumors, which are operated on, are small in size and the metastases are generally too disseminated to be considered operable. Therefore there is little material available for a systematic biochemical study, although such study appears necessary for several reasons. Results obtained with phenoloxidases (polyphenol-oxidase, catecholase, or tyrosinase) from plants or lower animals are generally considered valid for mammalian melanogenesis (*7*), yet several properties of these enzymes appear to be different from those of the similar enzyme of the transplantable mouse melanomas. Moreover, some of the properties of this "mammalian tyrosinase" are inconsistent with the very few data, existing in the older literature, that were obtained directly on human material. Alsberg (*8*) found that human melanoma extracts catalyzed the formation of melanin from catechol and possibly from tyrosine; Neuberg (*9*) reported that extracts of human melanoma catalyze pigment formation from tyramine and from epinephrine, but not from tyrosine. However, "mammalian tyrosinase," prepared from the Harding-Passey mouse melanoma, is reported (*6*) to be specific for 3:4-dihydroxyphenylalanine (dopa) and for tyrosine, and to have only a very slight action (in the absence of a considerable quantity of dopa) on all other dihydroxy- or monohydroxyphenols.

In recent years four human melanomas—one primary tumor and three relatively conspicuous metastatic masses—have been studied by the author. The present paper deals with the preparation of the human melanoma extracts and their activity in oxidizing dopa, tyrosine, and some other dihydroxy- and monohydroxyphenols, and also with the relationship of the oxidizing system of these human melanomas to copper and to some

[1] Received for publication July 27, 1953.

[2] A preliminary report was presented at the 2d International Congress of Biochemistry, Paris, July 1952 (Abstracts of Communications, p. 280).

other metals. The affinity for oxygen of the human melanoma enzyme is compared with that of a vegetal enzyme in the following paper.

Materials and Methods

The tumors, all classified histologically as highly malignant melanomas, were received from the operating room immediately after their removal, and were allowed to cool on ice to +2° C. All further operations were carried out in a cold room, at the same temperature, with apparatus and reagents previously chilled. The tumors were carefully dissected, weighed, and homogenized with an equal volume of 0.05 *M* Na_2HPO_4. A Waring blendor was used to homogenize the two larger metastatic masses (weighing 60 gm. and 115 gm. respectively), while the third metastatic mass (12 gm.) and the primary tumor (1.5 gm.) were ground with sand in a mortar. The coarse homogenate was strained through gauze, centrifuged for 5 minutes at 3,000 revolutions per minute and the precipitate discarded. This centrifugation leaves essentially all the activity in the supernatant but eliminates a great amount of inert material and renders exact pipetting possible. In all the following experiments the supernatant of this centrifugation was used. However, centrifugation for 30 minutes at 5,000 revolutions per minute sediments 70 percent of the activity.

Dialysis against pyrex-redistilled water was made in a cellophane bag (Visking cellulose sausage casing) against a 100-fold volume of water for 12 hours, followed by two exchanges against a 60-fold volume of water, each for 6 hours.

Dialysis against HCN was made by placing 20 ml. of the previously dialyzed extract and 5 ml. of 0.1 *M* KCN in a cellophane bag and dialyzing for 12 hours against 1,000 ml. of 0.1 *M* phosphate buffer at *p*H 7.4 containing 0.02 *M* KCN. This dialysis was followed by 2 exchanges each against 500 ml. of 0.01 *M* Na_2HPO_4 for 6 hours, 1,000 ml. for 12 hours, and 500 ml. for 6 hours. The dialyzed extract was brought to *p*H 6.8 with 0.1 *M* KH_2PO_4, and adjusted to twice its original volume before use.

The preparation of the vegetal phenoloxidase, used in the experiment reported in text-figure 4, was described earlier (*10*).

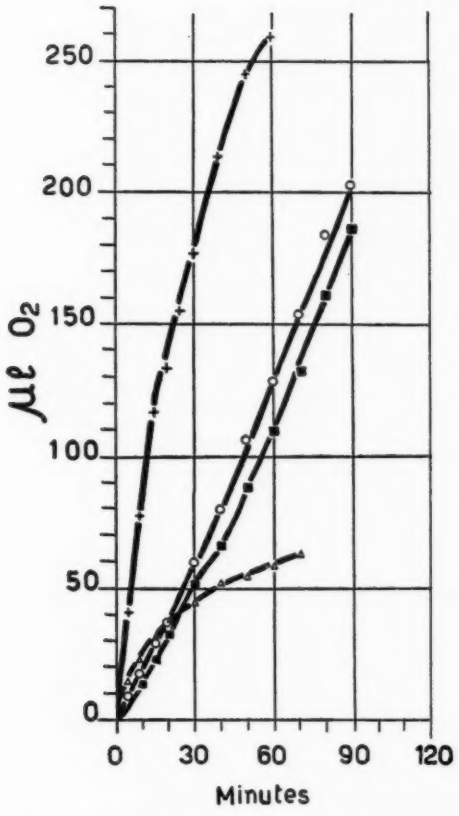
Copper was determined with sodium diethyldithiocarbamate, after the method of Keilin and Mann (*11*).

Oxygen consumption was measured in a Warburg apparatus, at 38° C. The total reaction volume was always 3 ml.; buffers were 0.05 *M* phosphate or, in the presence of excess metals, phosphate citrate at *p*H 6.8. Substrates were added from the side arms, after 15 minutes of equilibration. The center well of the flasks contained 0.10 ml. of 15-percent NaOH.

Throughout this work only pyrex-redistilled water was used, and the usual precautions were taken to eliminate trace metals from glassware and reagents.

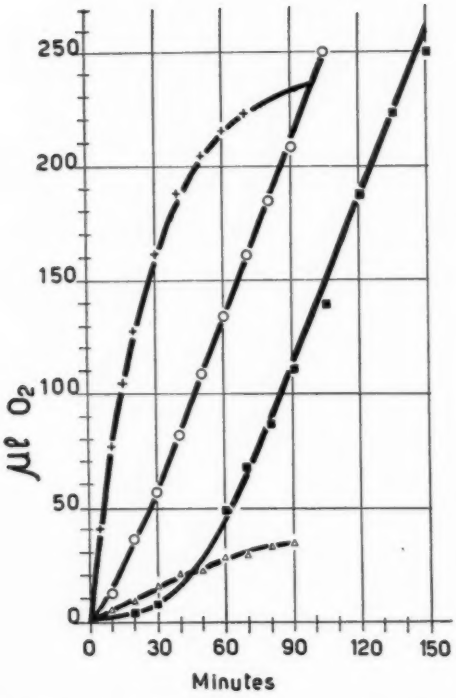
Results

The results obtained with the 60-gm. metastatic mass will be described first.



TEXT-FIGURE 1.—The action of a human melanoma homogenate (dry weight = 21.6 mg. of nondialyzable matter) on: +—1 mg. dopa; ■—1 mg. tyrosine; ○—0.98 mg. tyrosine + 0.02 mg. dopa; △—without substrate.

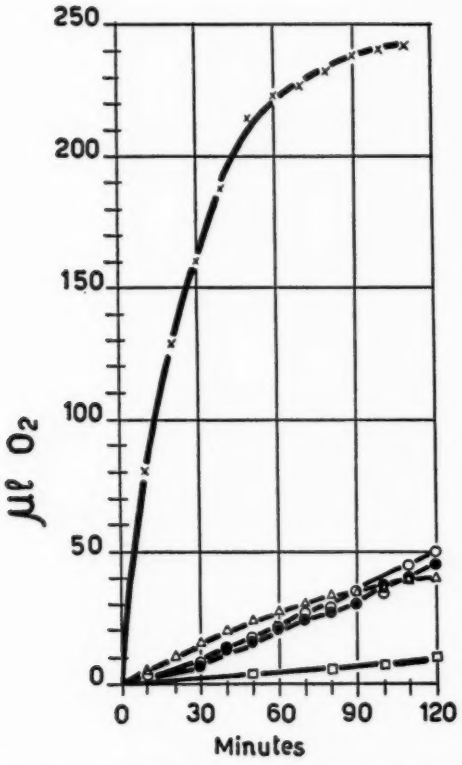
The activity of the homogenate on dopa, and on tyrosine in the absence and in the presence of a small amount of dopa, is reported in text-figure 1.



TEXT-FIGURE 2.—The action of a dialyzed homogenate of human melanoma (dry weight = 21.6 mg.) on: +—1 mg. dopa; ■—1 mg. tyrosine; ○—0.90 mg. tyrosine + 0.10 mg. dopa; △—without substrate.

Dialysis against water does not significantly change the activity on dopa and on tyrosine, but the induction period of the tyrosine oxidation is greatly prolonged (text-fig. 2). The autogenous respiration is greatly reduced by the dialysis.

The extract and the dialysate can be preserved at +2° C. in liquid state for several days. Repeated freezing (at −20° C.) and thawing do not alter their activity, and frozen extract and dialysate can be preserved for more than 2 years without any alteration.



TEXT-FIGURE 3.—The action of a dialyzed homogenate of human melanoma (dry weight = 21.6 mg.) on: ×—1 mg. dopa; ○—1 mg. epinephrine; ●—0.55 mg. catechol; △—without substrate; □—epinephrine alone, without homogenate.

The activity of the dialysate on dopa, epinephrine, and catechol is reported in text-figure 3. It is clear that the enzyme from human melanoma does not significantly oxidize the epinephrine or the catechol.

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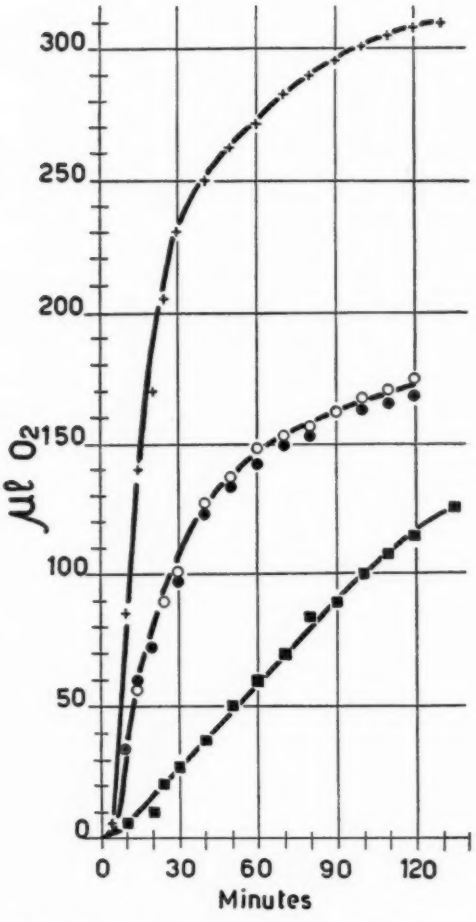
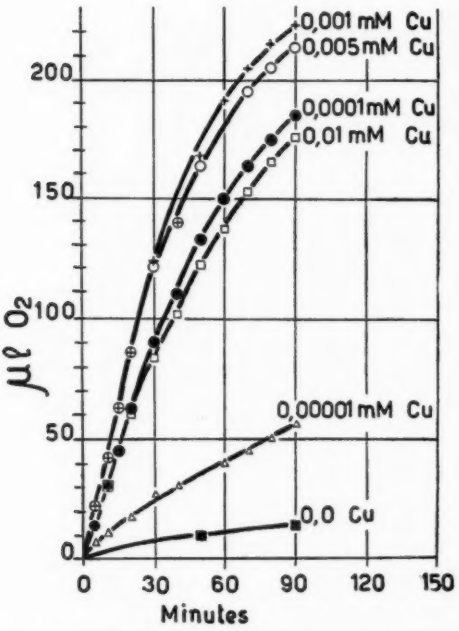


TEXT-FIGURE 4.—The action of potato phenoloxidase (12 enzyme units) on: ×—0.95 mg. tyramine-hydrochloride; ○—1.31 mg. glycyl-tyrosine; ●—1.42 mg. N-chloroacetyl-tyrosine; ■—1 mg. tyrosine.

The same amount of dialysate is completely inactive on tyramine, glycyl-tyrosine, and N-chloroacetyl-tyrosine. The fact (often ignored) that the enzyme of vegetal origin oxidizes these three monohydroxyphenols more rapidly than it does tyrosine itself is shown in text-figure 4.

The nondialyzable copper content of the human melanoma studied here seems to be relatively low. The quantitative determination of copper, in 0.50 ml. of dialysate containing 7.2 mg. of material, was unsuccessful; the colorimetric readings obtained were not significantly greater than the



TEXT-FIGURE 5.—The action of HCN-treated homogenate of human melanoma (dry weight = 10.8 mg.) on 1 mg. dopa, in the presence of various amounts of copper.

control readings. Since with the method used it was regularly possible to determine exactly 2.5 to 3 µg. Cu, it is possible to infer that the non-dialyzable copper content of this melanoma was below 0.04 percent.

However, the enzyme of human melanoma, like the enzymes of melanomas of mice, insects, and plants, is also a copper enzyme. Dialysis against HCN followed by dialysis against copper-free distilled water completely inactivates the enzyme, and the activity can be restored by adding copper (*12*). The dopa-oxidase activity of HCN-treated human homogenate in the presence of various amounts of copper is reported in text-figure 5. The activity on dopa increases rapidly with increasing amounts of copper: 70 percent activity is restored by 0.058 percent Cu, 100 percent activity by 0.58 percent. Clearly, the amount of copper required for the complete reactivation of the enzyme is much greater than the original copper content of the enzyme. A further excess of copper does not augment the activity on dopa; on the contrary, a great excess of copper presents a pronounced inactivating effect. The activating effect of copper is strictly specific: Co^{++}, Ni^{++}, Fe^{+++}, or Zn^{++} cannot replace copper. A great excess of Co and Ni has an inactivating effect distinctly lower than the inactivating effect given by excess of copper.

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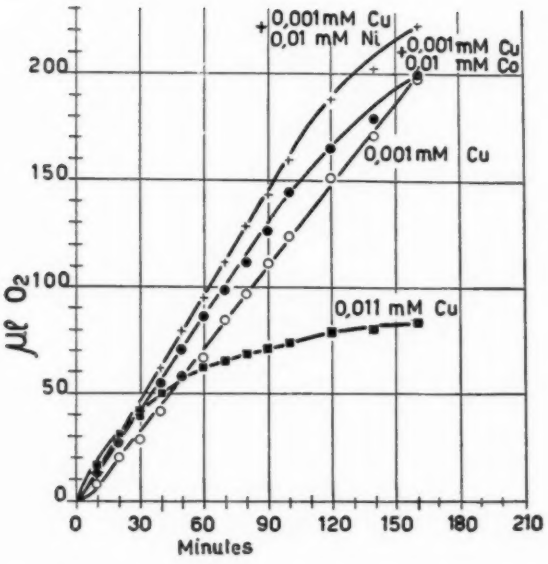

TEXT-FIGURE 6.—The action of HCN-treated homogenate of human melanoma (dry weight = 10.8 mg.) on 0.90 mg. tyrosine + 0.10 mg. dopa in the presence of copper, copper + excess cobalt, and copper + excess nickel.

The activity of the HCN-treated homogenate of human melanoma on tyrosine is reported in text-figure 6. To restore the activity on tyrosine the addition of a small amount of copper is necessary but not sufficient; it must be supplemented by a small amount of dopa. Excess cobalt or nickel, added after a small amount of copper, augment the activity on tyrosine. The activating effect of copper in excess is visible only during the first minutes of the tyrosine oxidation, as later the inactivating effect of excess copper counterbalances and exceeds its activating effect.

The results obtained with the 60-gm. metastatic mass were confirmed with the 12-gm. metastasis. However, dopa- and tyrosine-oxidase activities of this tumor were only one-third of the 60-gm. metastasis, measured on a dry weight basis.

The 1.5-gm. primary tumor and the 115-gm. metastasis showed a very low dopa-oxidase activity (about 25 times less than the 60-gm. metastasis) and were completely inactive on the tyrosine and on the other dihydroxy- or monohydroxyphenols tried. The small activity on dopa of the 115-gm. metastasis was completely eliminated by dialysis against HCN, and restored specifically by copper. The activity on dopa, tyrosine, and the other dihydroxy- or monohydroxyphenols was not increased by adding excess cytochrome C, vitamin B_{12}, Co^{++}, Ni^{++}, or Fe^{+++}.

Discussion

Although the four human melanomas examined here were histologically identical, their dopa-oxidase activity was found to differ widely. Dopa-oxidase activity and pigmentation are, to some degree, correlated in the S91 Cloudman melanoma; nonpigmented granules obtained from the amelanotic melanoma strain of Algire (*13*) appear to be incapable of oxidizing dopa (*3*). However, the four melanomas examined here appeared to be equally pigmented. The most active dopa-oxidase activity belonged to the most rapidly growing and most metastasizing of the four melanomas, but this can be due to mere chance. It is also possible that the pigment-forming enzyme system of melanomas can be more or less firmly bound to cellular architecture, and in certain cases it is destroyed by homogenization. This possibility, easy to control experimentally, must await new material for testing. In itself, a great variability in the oxidase activities of such heterologous material as human tumors is quite natural. The dopa- and especially the tyrosine-oxidase activities of the Cloudman and the Harding-Passey melanomas, although maintained in homologous strains of mice, were found to vary between wide limits (*3*).

The fact that certain human melanoma homogenates with low dopa-oxidase activity are incapable of oxidizing tryosine does not make it necessary to postulate the existence of a "tyrosinase" capable of oxidizing both tyrosine and dopa in the first two tumors, and a different "dopa oxidase" capable of oxidizing only dopa in the last two tumors discussed. Since it has been known for a long time that phenoloxidases in general, if strongly diluted, become incapable of oxidizing tyrosine (*14*, *15*), a different concentration of the enzyme could explain this apparent difference of specificity. The oxidation of tyrosine is a much more complicated process than that of dopa, and recently several arguments were presented (*10*, *16*) in favor of the old *o*-quinone theory of Onslow and Robinson (*17*), who proposed that the tyrosinase action is not due to the enzyme molecule itself, but to the *o*-quinones produced by the enzymic oxidation of the *o*-dihydroxyphenols. The significance, in favor of this theory, of the activating effect of certain metallic ions on the oxidation of the tyrosine by the enzyme of plant origin was amply discussed previously (*10*). It is shown in this paper that the same effect, although more difficult to demonstrate because of the greater inactivation of the human enzyme by an excess of the same metals, exists also in the case of the oxidation of tyrosine by the human melanoma enzyme.

In general, all properties of the phenol-oxidizing enzyme system of human melanomas, as described in this paper, appear identical to those of the transplantable mouse melanomas. Centrifugation of human melanomas shows that most of the oxidase activity is concentrated in a particulate fraction of the homogenate. The same fact was shown to hold for the Cloudman and for the Harding-Passey melanomas by DuBuy *et al.* (*3*), and by Lerner *et al.* (*4*). The effects of dialysis and freezing on human

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melanoma homogenate are identical to their effects on the homogenate of the Harding-Passey melanoma (*4*). The strict specificity of the Harding-Passey melanoma enzyme for dopa and for tyrosine, found by Lerner *et al.* (*6*), is identical to the strict specificity of the human melanoma enzyme for these two substances. Copper was recognized as a prosthetic group of the mouse melanoma enzyme by Lerner *et al.* (*5*). The same authors observed the important fact that the addition of copper alone restores the activity of the HCN-treated homogenate on dopa, but to restore the activity on tyrosine a small amount of dopa must also be added. It was shown in this paper that this behavior is exactly duplicated by the human melanoma homogenate.

The phenoloxidase found in human and mouse melanomas, like all other phenoloxidases, is a copper enzyme (*18*). It is interesting to note that HCN-treated homogenate of human melanoma, like vegetal phenoloxidase (*12, 19*), requires much more copper for complete reactivation than it contained originally. However, phenoloxidase from human and mouse melanomas appears profoundly different from all other phenoloxidases. This difference is not limited to the physical state of the enzyme, which is associated with particulate matter in the tumor homogenates and in free solution in the extracts prepared from plants. The principal difference lies in the strict specificity of the mammalian melanoma enzyme for dopa and for tyrosine. Warburg (*20*) states that potato phenoloxidase oxidizes catechol 1,000 times more rapidly than it does dopa and epinephrine; the human melanoma enzyme, like the mouse melanoma enzyme, does not oxidize any *o*-dihydroxyphenol except dopa. In the presence of the vegetal enzyme, all the other monohydroxyphenols are oxidized more rapidly than tyrosine, yet in the presence of the melanoma enzyme, tyrosine is the only monohydroxyphenol that is oxidized.

Summary

The phenol-oxidizing enzyme system of four human melanomas was studied.

Dopa- and tyrosine-oxidase activity of the tumors was found to vary between wide limits; two tumors of low activity on dopa were incapable of oxidizing tyrosine.

The enzyme system is bound to the particulate elements of the homogenate, and it is highly specific for dopa and tyrosine. Catechol, epinephrine, tyramine, glycyl-tyrosine, and N-chloroacetyl-tyrosine were not oxidized by human melanoma homogenate. With vegetal phenoloxidase, the last three monophenols were oxidized much more rapidly than tyrosine.

Human melanoma homogenate required copper for activity; removal of copper resulted in loss of the activity. The activity on dopa could be specifically restored by adding copper, although excess copper had a pronounced inactivating effect. Copper did not restore the activity on tyrosine unless a small amount of dopa was also added. The activity of the reconstituted enzyme on tyrosine was augmented by excess nickel and cobalt.

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The Relative Oxygen Affinity of Human and of Vegetal Phenoloxidase [1, 2]

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It was shown in the preceding paper (*1*) that the phenol-oxidizing enzyme system of human melanoma presents a substrate-specificity which differs profoundly from that of vegetal polyphenoloxidase. Since molecular oxygen can be considered the substrate of an oxidase, it seemed interesting to compare the relative specificities of the human and of the vegetal enzyme for molecular oxygen.

Materials and Methods

The uptake of oxygen was measured in a Warburg apparatus, at 38° C. The total reaction volume was always 3 ml.; 0.05 *M* phosphate, at *p*H 6.8, was used as buffer. After 15 minutes of temperature equilibration, 1 mg. of dopa (0.50 ml. of a solution of 2 mg. per ml.) was added from the side arm. The center well of the flasks contained 0.10 ml. of 15-percent NaOH.

The preparation of the homogenate of human melanoma was described in the preceding paper (*1*). A volume of 1.5 ml. of the dialyzed homogenate, prepared from the 60-gm. metastatic mass, was used for each flask. This amount of homogenate, of a dry weight of 21.6 mg., corresponds to 8.8 enzyme units, as defined by Hogeboom and Adams (*2*).

The preparation of the potato polyphenoloxidase apo-enzyme was described earlier (*3*). A dilute solution was used, containing 8 mg. of nondialyzable matter per ml. of the diluted solution. The minimal amount of copper necessary for the full activation of the apo-enzyme on dopa, which previously was shown to correspond to 0.08 percent (*3*), was added as $CuSO_4$.

The Warburg flasks, one containing the human and the other the vegetal enzyme, were always handled in parallel fashion; they were filled and saturated with the same mixture of nitrogen and oxygen, and agitated side by side at the same rapid rate (120 strokes per minute). Augmentation of this speed did not increase the rate or the amount of the oxygen uptake. Readings were made at 5-minute intervals. The

[1] Received for publication July 27, 1953.

[2] A preliminary report was presented before the 2d International Congress of Biochemistry, Paris, July 1952 (Abstracts of Communications, p. 280).

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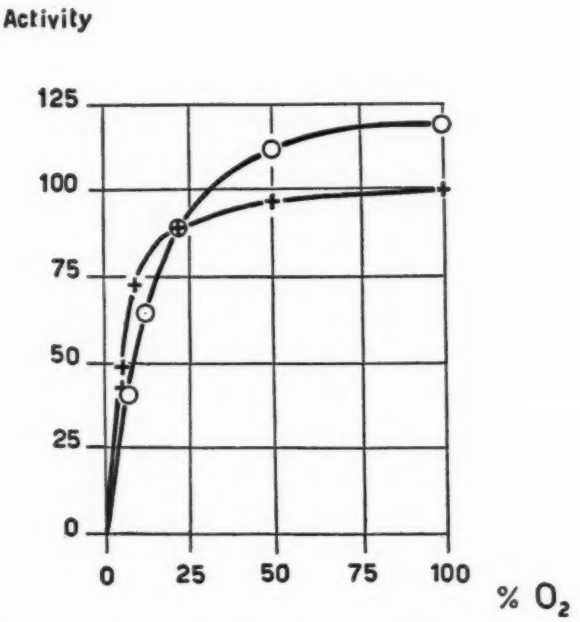
activities are expressed as μl. O_2 per minute, calculated from the value of the first reading.

Results and Discussion

In the first series of experiments, 0.30 ml. of the solution of potato enzyme, corresponding to 8.8 enzyme units, was used so that the activities of the human and of the potato enzyme would be equal in air. The activities of the two enzymes as a function of oxygen tension are reported in text-figure 1. The activity of the human enzyme increases relatively little (by 12%) on augmentation of the oxygen tension from 21 percent to 100 percent; the increase in activity of the potato enzyme is much more



TEXT-FIGURE 1.—The activities of the phenoloxidase in human melanoma homogenate (+) and an equivalent amount of potato phenoloxidase (○) on 1 mg. dopa, as a function of the O_2 tension. (The activities are expressed as μl. O_2 per minute, calculated from the value of the first reading.)

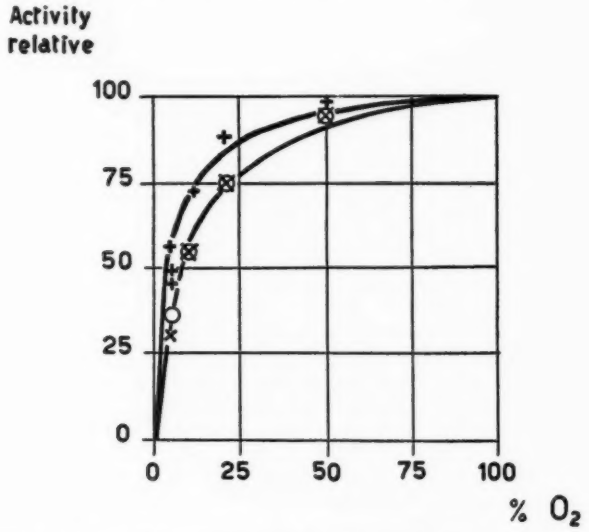
pronounced (25%). The behavior of the two enzymes also remains different when the oxygen tension is lowered; the activity of the vegetal enzyme decreases more rapidly than that of the human enzyme.

The comparison of the two enzymes becomes clearer when the relative activities are calculated, taking the maximum activity in 100-percent oxygen as 100-percent activity. The results of this calculation are reported in text-figure 2. The calculated values were verified experimentally, by using the same amount of human enzyme as in the first series of experi-

ments, but only 0.225 ml. of the potato enzyme. The reproducibility of the measurements is excellent between 100 percent and 10 percent O_2. Working with 5 percent O_2, the measurements can be reproduced with an error of ± 5 percent. However, at a 2.5-percent oxygen tension the individual measurements can differ as much as 30 percent; the latter values were therefore neglected. It is shown in text-figure 2 that the experimental points lie on or close to the theoretical curves for the equation

$$A = \frac{100P}{K_1 + K_2P} \tag{1}$$

where A is the activity, P is the percent of oxygen tension, and K_1 and K_2 are constants. For the potato enzyme, $K_1=9$ and $K_2=0.915$; for the human enzyme, $K_1=4.5$ and $K_2=0.96$.



TEXT-FIGURE 2.—The points represent the relative activities of human melanoma enzyme (+) and potato phenoloxidase (○ and ×) on 1 mg. dopa, as a function of the O_2 tension. The curves represent the theoretical curves from equation (1).

Plotting 100 P/A against P (text-fig. 3), linear curves are obtained, expressing the equation

$$100\ P/A = K_1 + K_2P \tag{2}$$

The interpretation of these equations, which describe the rate of oxygen consumption by numerous organisms and tissue slices as a function of the oxygen tension, was adequately discussed by Tang (*4*). That author has already pointed out the close relationship of these equations to the well-known equation of Hill and Redfield for the oxygen-dissociation curve of hemoglobin and of hemocyanin. He also pointed out the possibility of

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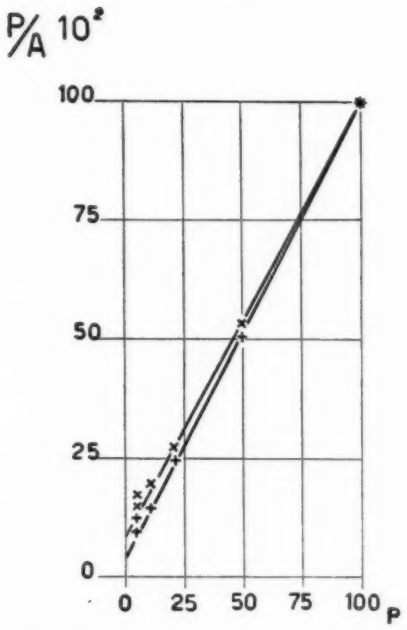

TEXT-FIGURE 3.—100 P/A plotted against the O_2 tension (equation 2). +—human melanoma phenoloxidase; ×—potato phenoloxidase.

transforming these equations by transforming the constants in the equation of Warburg and Kubowitz for cell respiration (*5*). It is evident that the curves of text-figures 1 and 2 are typical Michaelis curves, and that the linear curves of text-figure 3 correspond to the familiar expression of Lineweaver and Burk (*6*) for the equation of Michaelis and Menten (*7*):

$$S/v = S/V + K_s/V \tag{3}$$

In fact the oxygen tension P corresponds to the substrate concentration S, activity A to the rate v, the maximum rate of oxidation in the presence of excess substrate V is equal to $100/K_2$. K_s is, evidently, equal to K_1/K_2.

The Michaelis constant for the oxygen of the potato phenoloxidase is approximately twice that of the human enzyme. The latter is 50-percent saturated in the presence of 4.7 percent O_2, the former requires 9.8 percent O_2 for 50-percent saturation. Szent-Györgyi and Vietorisz (*8*) observed the sudden augmentation of the rate and amount of oxygen uptake of potato tissue at the moment of an injury to the cells, such as from grinding; the same injury also initiates the brown discoloration. These facts were explained by supposing that phenoloxidase and phenolic substrates are separated spatially in the intact cells, and that they are allowed to come in contact by the injury. This explanation seems probable, considering not only the phenols but also atmospheric oxygen as a substrate of the enzyme.

In the case of intact tissues, the oxygen substrate and the enzyme are in a sense separated, or more exactly, their surface of contact increases enormously at the moment of injury to the cells. Mammalian melanoma tissues need not be injured or ground to become black. Since they have an efficient system of oxygenation, they certainly possess more oxygen than do potato cells; moreover, their enzyme is relatively more efficient at lower tensions of oxygen.

The Michaelis constants reported here are only relative; they express the affinity for oxygen of the human enzyme, in the presence of a certain amount of dopa and at 38° C., relative to the affinity for oxygen of the potato enzyme under the same conditions. For the human enzyme, absolute values cannot be calculated because the concentration of the enzyme, that is the quantity of copper fixed on the catalytically active places of the enzyme protein, is unknown. This quantity is known, however, for the potato phenoloxidase used here, although only very approximately; it was shown previously that this partially purified apo-enzyme is saturated at a copper content of 0.08 percent. The constants, after Warburg (*9*) and Kubowitz (*10*), considering that 3 ml. of reaction volume contain 1.8 mg. of protein corresponding to 1.44 μg. of copper (0.75×10^{-5} M per liter) and 1 mg. (1.68×10^{-3} M per liter) dopa, are:

$$k_{ox} = 6.2 \times 10^5 \frac{1}{\frac{Mole\ O_2}{Liter}\ minutes}$$

and

$$k_{red} = 4.89 \times 10^4 \frac{1}{\frac{Mole\ dopa}{Liter}\ minutes}$$

After Warburg (*9*) and Kubowitz (*10*), the corresponding values for catechol are:

$$k_{ox} = 1.41 \times 10^8 \frac{1}{\frac{Mole\ O_2}{Liter}\ minutes}$$

and

$$k_{red} = 5.9 \times 10^7 \frac{1}{\frac{Mole\ catechol}{Liter}\ minutes}$$

Comparison of the two k_{red} values confirms the statement of these two authors that potato phenoloxidase oxidizes catechol 1,000 times more rapidly than it does dopa. It was shown in the preceding paper (*1*) that human melanoma enzyme does not significantly oxidize catechol.

Summary

The dopa-oxidase activities of equivalent amounts of human melanoma homogenate and a vegetal phenoloxidase were measured at different tensions of oxygen. Typical hyperbolic saturation curves were obtained,

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which permitted calculation of the relative affinity of the human and of the vegetal enzyme for oxygen. The affinity of the vegetal enzyme is about half that of the human enzyme. Some implications of this finding are discussed.

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Fractionation Studies on the Tumor-Necrotizing Agent from *Serratia marcescens* (Shear's Polysaccharide)[1,2]

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Systemic injections of various bacterial filtrates are known to produce hemorrhagic necrosis in animal tumors (*1–4*), while normal tissues remain largely unaffected. Coley (*5*) employed crude filtrates from two species of bacteria in the treatment of cancer patients. This subject was reviewed by Shear and Perrault (*3*). In 1936, Shear and collaborators began to purify the factor in the bacterial filtrates and developed a satisfactory bioassay method (*6–10*). They isolated a highly active tumor-necrotizing material from filtrates of cultures of *Escherichia coli* and of *Bacillus prodigiosus*. This material was further purified by trypsin digestion without loss of activity. About two-thirds of it was considered to be polysaccharide in nature. After hydrolysis this material yielded aldohexose, hexosamine, methylpentose, and the components of a phospholipid. The lipid was firmly linked to the carbohydrate moiety and could not be removed without inactivation. They concluded that the active material was a lipopolysaccharide.

Recently Ikawa *et al.* (*11*) reported further results of an investigation of the tumor-hemorrhagic agent from cultures of *E. coli*. They isolated a lipopolysaccharide after removing most of the peptide components by treatment with trichloroacetic acid and subsequent fractionation with ethanol. From their results it would appear that the peptide component is not essential for the hemorrhagic effect of the lipopolysaccharide; it was observed, however, that the removal of the peptide fragments resulted in decreased water-solubility.

The interest in lipid-bound polysaccharides has increased since Haworth, Kent, and Stacey (*12*) isolated an antigenic complex by extracting heat-killed tubercle bacilli with urea or β-hydroxypropionamidine. The fractions that were serologically most active contained a lipid, desoxyribonucleic acid, and a serologically specific polysaccharide containing D-galactopyranose, D-mannopyranose, D-glucosamine and D-arabofuranose, and having properties similar to a lipid-bound polysaccharide

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isolated by alkaline extraction from *Mycobacterium tuberculosis* cells. Other studies on bacterial polysaccharides may also be mentioned (*13–16*).

The present paper reports the results of an investigation on the fractionation of a preparation from the cells of *Serratia marcescens* (Shear's polysaccharide), which produces hemorrhagic necrosis in tumors (*17*), and on the composition of the starting material and its fractions. Various methods for the fractionation and analysis of the active material were employed, and were modified according to experience.

Material and Methods

The starting material for the present investigation was provided by Dr. M. J. Shear of the National Cancer Institute. It was designated Lot No. P-25 of a polysaccharide-containing fraction isolated from the cells of *S. marcescens* by extraction with trichloroacetic acid and phenol. The extract had been further fractionated by Perrault and Shear in much the same way that they had previously treated the material isolated from the filtrates of the bacterial cultures. All data herein reported are calculated on the basis of freeze-dried material.

The reducing power was determined according to the Hagedorn-Jensen method [modification of Hanes' method (*18*)], and expressed in percent glucose.

Hexosamine and N-acetylhexosamine.—Hexosamine was assayed by the method of Elson and Morgan [modification of the method of Palmer *et al.* (*19*)]. Glucosamine hydrochloride concentrations between 10 and 50 μg. per ml. were used as standards. The optical densities of the test solutions and the standards were determined in a Beckman spectrophotometer at 5,400 Å. N-acetylhexosamine was assayed by the method of Morgan and Elson (*20*) and the colorimetric readings were carried out as above. N-acetylglucosamine concentrations between 10 and 40 μg. per ml. were used as standards.

Acetyl content.—The method of Kuhn and Roth (*21*), as described by Grant (*22*), was used. Preliminary assays indicated that the release of acetyl groups was complete after a heating period of 3 hours with 25 percent toluenesulfonic acid at 100° C. The results thus obtained represent the total of volatile acids, expressed in percent CH_3CO, including the fatty acids from the phospholipid.

Phosphorus content.—The sample was digested with 20 *N* sulfuric acid, containing 3 percent of concentrated nitric acid, by heating over a small flame until sulfur trioxide fumes evolved. After neutralizing with sodium hydroxide until phenolphthalein just turned red, phosphorus was determined according to the method of Fiske and Subbarow (*23*).

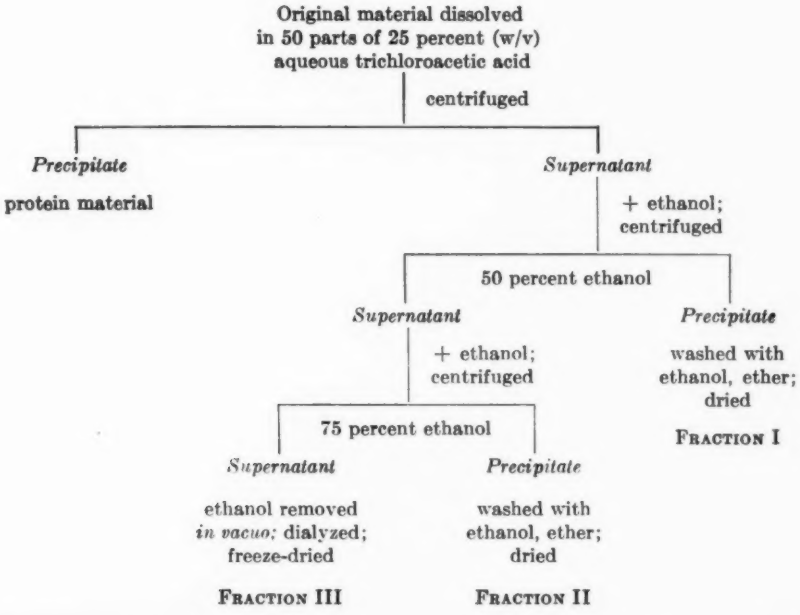
The nitrogen content was estimated by the Kjeldahl method, using a boric acid–methyl red mixture. The nitrogen content of some preparations was checked by the Dumas micromethod.

α-Amino-nitrogen was determined by measuring the nitrogen liberated after treatment with HNO_2 in the Van Slyke manometric apparatus (*24*). For this purpose the material was hydrolyzed with 6 *N* hydrochloric acid

and aliquots were withdrawn at intervals. The hydrochloric acid was removed *in vacuo* over solid potassium hydroxide and concentrated sulfuric acid before proceeding with the nitrogen analysis. If the hydrochloric acid is not removed but neutralized, the values obtained are not reliable.

Hydrolysis.—The samples were hydrolyzed with 1.5 *N* sulfuric acid, 1.5 *N* and 6 *N* hydrochloric acid, or *N* acetic acid, in sealed glass ampules that were heated for an appropriate length of time on a boiling water bath. In order to avoid side reactions, all hydrolyses were carried out in a nitrogen atmosphere. The ampules were then cooled. When the hydrolysis had been carried out with hydrochloric acid, the resulting mixture was evaporated to dryness *in vacuo* over solid potassium hydroxide and concentrated sulfuric acid. When acetic or sulfuric acid had been used, the mixture was neutralized with sodium hydroxide.

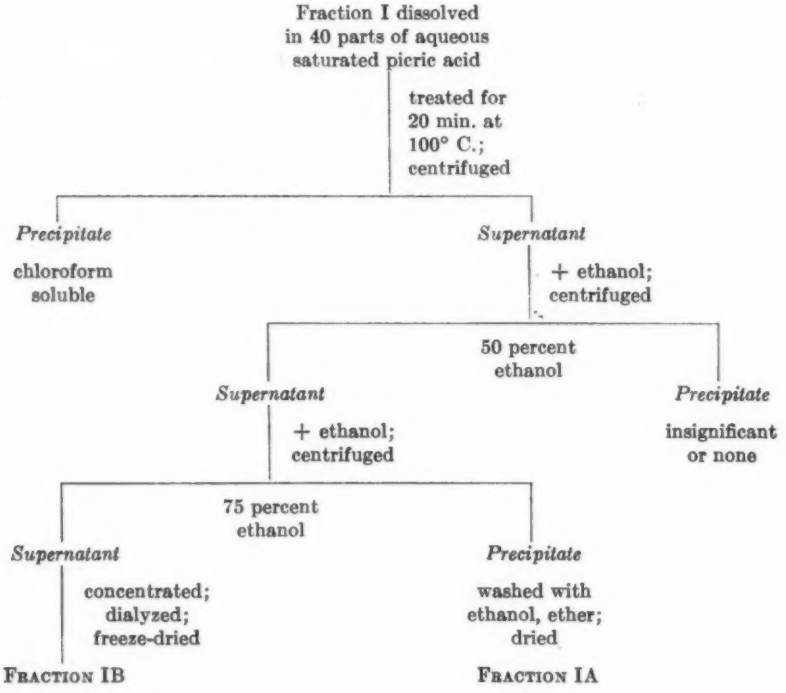
Fractionation of the starting material.—In preliminary experiments it was established that almost all the active material is precipitated from an aqueous solution of the starting material by the addition of one volume of ethanol. Prior to fractionation with ethanol, the material was treated with 25 percent trichloroacetic acid in order to remove any free protein material present. A similar procedure has been employed for the fractionation of the hemorrhagic agent from *E. coli* (*11*). The fractionation procedure is given in text-figure 1. Analytical data relating to the fractions thus obtained are given in table 1.



TEXT-FIGURE 1.—Treatment of Shear's polysaccharide with trichloroacetic acid and subsequent fractionation with ethanol.

Cleavage of Fraction I.—Goebel, Binkley, and Perlman (*25*), when investigating the somatic antigen of *Shigella paradysenteriae*, dissociated the protein-polysaccharide-lipid complex by heating with saturated picric acid solution. This reagent was equally effective with Fraction I.

Fifty mg. of Fraction I were dissolved in 2 ml. of saturated aqueous picric acid solution and heated for 20 minutes on a boiling water bath. The mixture was then centrifuged and the residue washed with water and acetone and then dissolved in chloroform. The supernatant was fractionated according to the procedure shown in text-figure 2. Analytical data relating to the water-soluble fractions are given in table 2.



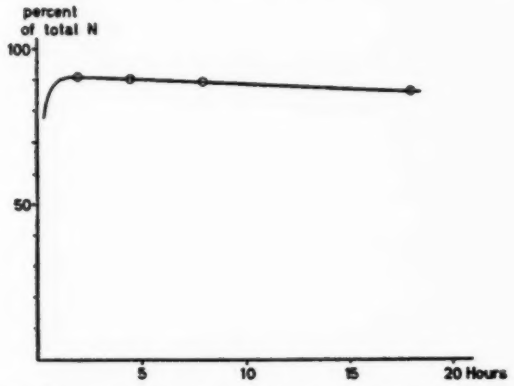
Text-figure 2.—Cleavage of Fraction I with picric acid and subsequent fractionation with ethanol.

Results

Acid Hydrolysis of the Tumor-Hemorrhagic Agent

Hydrolysis with 6 N hydrochloric acid gave rise to considerable decomposition of the material with the formation of a black precipitate of humic acids, reaching 15 percent of the total amount after 12 to 20 hours of heating.

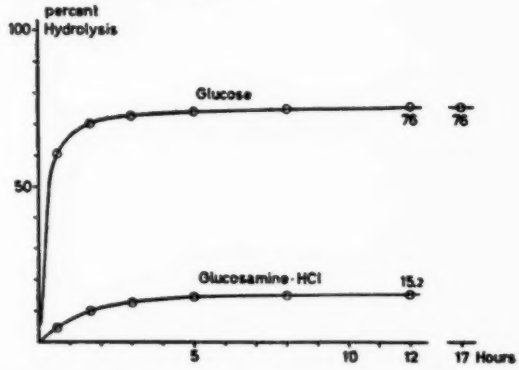
The course of the hydrolysis was followed by measuring the amount of liberated α-amino-nitrogen in the Van Slyke manometric apparatus.



TEXT-FIGURE 3.—The liberation of α-amino groups during hydrolysis of Shear's polysaccharide with 6 *N* hydrochloric acid at 100° C., expressed in percent of the total nitrogen.

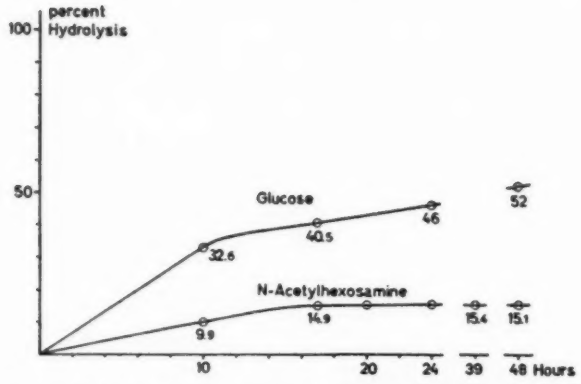
The maximum value was reached in about two hours and represented about 91 percent of the total nitrogen content (text-fig. 3).

Hydrolysis with 1.5 N *sulfuric acid* was followed by the determination of the liberated reducing substances and hexosamine. The maximum value for reducing sugars was reached after 12 hours, and represented 76.3 percent dry weight. The maximum liberation of hexosamine, 15.2 percent, expressed as glucosamine hydrochloride, occurred at approximately the same time (text-fig. 4).



TEXT-FIGURE 4.—Reducing power (expressed as glucose), and hexosamine (expressed as glucosamine hydrochloride) during hydrolysis of Shear's polysaccharide with 1.5 *N* sulfuric acid at 100° C.

Hydrolysis with N *acetic acid* was followed by the estimation of the reducing power and of N-acetylhexosamine. The maximum value for N-acetylhexosamine was reached after 24 hours and represented 15.4 percent, expressed as N-acetylglucosamine base. The results indicated that all the hexosamine present was N-acetylated (text-fig. 5).



TEXT-FIGURE 5.—Reducing power (expressed as glucose) and N-acetylhexosamine (determined as N-acetylglucosamine base) during hydrolysis of Shear's polysaccharide with *N* acetic acid at 100° C.

Fractionation Experiments

The fractionation of the starting material according to the method described earlier (text-fig. 1) yielded three fractions. Shear and Perrault found only Fraction I to be active.[4] Fraction I gives a turbid solution when dissolved in water. The analytical data reported in table 1 show that it contains less acetyl and less hexosamine than the starting material; other data remained essentially unchanged. Fraction I gives a negative Tollens test for pentoses and methylpentoses, and a negative Dische test for desoxyribose. Dische's hexuronic acid test (*26*) was weakly positive; less than 2 percent of hexuronic acid was found. Ultraviolet absorption spectra did not show any specific absorption in the range of nucleic acids

TABLE 1.—*Analytical data on the fractions obtained by treatment of the starting material with trichloroacetic acid and subsequent fractionation with ethanol*

	Starting material	Fractions		
	(percent)	I (percent)	II (percent)	III (percent)
Yield	.....	65–70	13	10
Reducing power (in percent glucose) on optimum hydrolysis	76. 3	77	82. 3	33
Glucosamine hydrochloride	15. 2	10. 6	19. 5	9. 1
N-acetylglucosamine base	15. 4	10. 8		
Glucuronic acid	<2. 0	<2. 0		
Nitrogen (Kjeldahl)	1. 81	1. 80		
Nitrogen (Dumas)	1. 87	1. 87		
α-Amino-nitrogen	1. 64	1. 60		
Acetyl	4. 30	3. 85	6. 1	
Sulfur*	<0. 1	<0. 1		
Phosphorus	1. 1	1. 1		
Tumor-necrotizing activity†	positive	positive	negative	negative

*Determined by S. Paulson (*87*).
†Personal communication from M. J. Shear and A. Perrault.

[4] Personal communication from Dr. M. J. Shear.

Cleavage of Fraction I

The cleavage of Fraction I with saturated aqueous picric acid solution followed by fractionation with ethanol (text-fig. 2) yielded two main carbohydrate fractions, IA and IB. Analytical data are given in table 2.

TABLE 2.—*Analytical data on the fractions obtained by treatment of Fraction I with saturated picric acid and subsequent fractionation with ethanol*

	Fractions		
	I	IA	IB
Yield (percent dry weight)	.....	40	25–30
Reducing power (as percent glucose on optimum hydrolysis)	77	91	67
Glucosamine hydrochloride (percent d. w.)	10. 6	3. 6	18. 5
N-acetylglucosamine base (percent d. w.)	10. 8	3. 6	18
Hexuronic acid (percent d. w.)	<2. 0	<2. 0	
Phosphorus (percent d. w.)	1. 1	0. 2	0. 6
Tumor-necrotizing activity*	positive	negative	negative
Optical rotation $[\alpha]_{D}^{20}$	+136°	+193°	

*Personal communication from M. J. Shear and A. Perrault.

These analytical data indicate that Fraction IA consists almost completely of glucose-containing material. Most of the hexosamine was found in Fraction IB; N-acetylhexosamine estimation on this fraction indicates that the N-acetyl groups remained intact after picric acid treatment. It is important to note that after treatment with picric acid no fraction precipitating with 50 percent ethanol was obtained (*cf.* text-fig. 2). All fractions obtained were inactive (*cf. 9*) and precipitated at levels of 75 percent ethanol and higher. The water-insoluble residue obtained in a yield of about 25 percent (w/w) contained mainly lipid material and protein picrate; it still gave a positive anthrone test for carbohydrates. It was soluble in chloroform and insoluble in water, acetone, and ethanol. This material was not further analyzed.

Discussion

Our results indicate that the tumor-hemorrhagic material in Shear's polysaccharide is a phospholipid-polysaccharide-protein complex. The cleavage of this complex with picric acid resulted in its separation into a water-soluble and a water-insoluble moiety, and was followed by a complete loss of its tumor-necrotizing properties. The water-soluble moiety, after fractionation with ethanol, contained no material precipitating at the level of 50 percent ethanol. This suggests a complex-like structure of the tumor-necrotizing material and indicates that the components after chemical isolation are inactive as such. The varying proportions of N-acetylhexosamine and hexose found in these carbohydrate fractions, and additional data presented in the following paper (*28*), further suggest that two different polysaccharides are present: a glucan, and a polysaccharide containing N-acetylhexosamine. It is not yet estab-

lished whether the latter is built up of N-acetylhexosamine only, or whether it also contains hexose similar to the type IV pneumococcus-specific polysaccharide. Hexuronic acid was found in amounts of less than 2 percent and cannot therefore be considered a main constituent of either of them. Fraction IA, consisting almost entirely of a glucan, as shown in the following paper (*28*), does not stain with iodine and is therefore different from the polysaccharides of the glycogen and starch groups. The high positive specific optical rotation in water and the decrease in rotation observed during hydrolysis suggest that the glucosidic linkages may have the α-configuration.

The glucan in question may provisionally be regarded as storage material built up from the excess of glucose present in the culture medium of *Serratia marcescens*. Gale (*29*) previously noted that bacteria accumulate reserves of polysaccharide material within the cell. When, for instance, *E. coli* is allowed to metabolize glucose in excess, polysaccharide formation occurs within the cell. Doudoroff *et al.* (*30*) and Torriani and Monod (*31*) found, furthermore, that the polysaccharide formed did not stain with iodine if the growth medium contained excess of glucose.

The water-insoluble moiety obtained during picric acid treatment is mainly composed of lipid material and protein picrate, and still contains a small amount of carbohydrate. Bioassays carried out on this fraction have been negative. It is possible that the lipid moiety has undergone a change in boiling picric acid solution that led to its inactivation (*cf. 9*). This question however remains unsolved until a more gentle means of dissociating the complex is found. The active Fraction I still contains firmly linked protein material that could not be removed by treatment with trichloroacetic acid. It is possible that this firm linkage is due to denaturation of the protein fragment during the extraction and purification procedures.

Summary

Fractionation experiments on the material from cells of *Serratia marcescens* (Shear's polysaccharide), which induces hemorrhagic necrosis in animal tumors, are described. The active substance was found to be a phospholipid-polysaccharide-protein complex. The acid hydrolysis products contained 77 percent of reducing substances, expressed as glucose, and 11 percent of N-acetylhexosamine. The α-amino-nitrogen represented 91 percent and the α-amino-acid nitrogen about 50 percent of the total nitrogen (1.8 percent), corresponding to a protein content of 5 to 6 percent. The phospholipid content varied from 11 to 15 percent. The carbohydrate moiety probably consists of two polysaccharides: a glucan, and a polysaccharide containing N-acetylhexosamine as its main constituent.

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Chemical Studies on the Tumor-Necrotizing Agent from *Serratia marcescens* (Shear's Polysaccharide)[1,2]

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In a previous paper (*1*) we reported further fractionation experiments and analytical data on the extracted material (Lot No. P-25) from cells of *Serratia marcescens* (Shear's polysaccharide), which induces necrosis in animal tumors. Physicochemical data obtained by Malmgren (*2*) show that this preparation has a smaller particle size and an appreciably higher electrophoretic mobility than earlier preparations investigated by Kahler, Shear, and Hartwell (*3*). The purified material, as revealed by degradation experiments, was characterized as a *phospholipid-polysaccharide-protein complex*, the carbohydrate moiety representing about 76 percent of the total dry weight (*1*).

The present paper reports further investigations on the properties of the purified material and on the nature and composition of its protein and polysaccharide components. It was found that the suggested storage material, a glucan, can be largely removed by digestion with a crude soybean β-amylase preparation. The remaining part of the complex was found to be more active than the starting material. The lipid part was not further analyzed.

Material and Methods

Material.—For the present investigation, Fractions I and IA of Shear's polysaccharide (Lot No. P-25) were used; they were obtained by fractionation of the starting material according to the methods described in the preceding paper (*1*). All quantitative data reported herein are calculated on the basis of freeze-dried material.

Chromatographic methods.—Paper chromatography was used for the examination of amino acids, hexosamines, and sugars and was employed as described by Consden, Gordon, and Martin (*4*).

Amino acids were examined qualitatively on the two-dimensional chromatogram run with collidine and phenol as solvent systems. The amino acids were detected by spraying the paper sheet with ninhydrin reagent and drying it at 85° C. for 5 minutes (text-fig. 1).

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Sugars and hexosamine were examined on the one-dimensional chromatogram, run with phenol or collidine as solvents. The sugar spots were detected by spraying with aniline-oxalate reagent and subsequently heating the paper strips for 10 minutes at 110° C. The hexosamines were identified by spraying with alkaline acetylacetone followed by *p*-dimethylaminobenzaldehyde reagent (*5*).

For the *quantitative estimation of the sugars and hexosamines*, one-dimensional chromatograms were run, using propanol:water (6:4) as a solvent system. For these chromatograms, the paper sheets were rolled into cylindrical form and allowed to irrigate by the ascending technique of Williams and Kirby (*6*).

After irrigation, the solvent was removed from the chromatograms by evaporation overnight in a circulating air oven at 30 to 35° C. One strip was cut off and developed by spraying with aniline-oxalate reagent. The remaining sheet was divided into corresponding zones by reference to the small strip. Equal paper areas were cut from the major sheet to serve as paper blanks. The strips were then eluted with water in a small Soxhlet's apparatus. The reducing power of the extracts and of an aliquot of the original hydrolysate was then estimated according to the ferricyanide method of Park and Johnson (*7*), and expressed in percent glucose.

The recovery of the sugars varied between 84 and 90 percent. From the sugar ratios thus obtained, the relative amounts of sugars present were estimated. Hexuronic acid estimations on sugar extracts were carried out according to Dische's carbazol method (*8*).

The identification of the methylated sugars was carried out according to methods described previously (*5*, *9*). The solution of methylated sugars under investigation was examined on the paper chromatogram using as a solvent the top layer of a mixture of *n*-butanol (40 percent), ethanol (10 percent), water (49 percent), and ammonia (1 percent). Tetramethyl D-glucose was used as a reference sugar. The paper sheet was then dried and sprayed with an ammoniacal solution of silver nitrate (10 percent); on heating, the positions of the separated sugars were indicated by brown spots. The R_G values of the separated sugars were then calculated from the ratio between the distance from the starting line to the center of the spot and the distance through which the tetramethyl D-glucose had moved. The R_G values of the separated components were compared to those given by Hirst, Hough, and Jones (*9*); further evidence of identity was then obtained by comparison on another chromatogram with an artificial mixture of the suspected sugars.

Methylation, methanolysis, and hydrolysis of the carbohydrate moiety.—Eighty mg. of Fraction IA were methylated for 6 hours with dimethylsulfate and aqueous sodium hydroxide at 45° C. in a nitrogen atmosphere, maintaining a *p*H of 12, with alizarin yellow R as an indicator. After elimination of the salts by a 24-hour dialysis against distilled water, the solution was concentrated *in vacuo* to a small volume. This methylation procedure was repeated 6 times consecutively. After the seventh methylation the solution was dialyzed, electrodialyzed, and then freeze-dried.

The examination of the methylated polysaccharide was then carried out according to the general technique of Hirst, Hough, and Jones (*9*) as follows:

Fifty mg. of the methylated polysaccharide were hydrolyzed with 4 percent methanolic hydrogen chloride (1 ml.) in a sealed tube at 100° C. for 20 hours. After hydrolysis, the tube was opened and the methanol removed by boiling. An aqueous solution of hydrochloric acid (4 percent, 5 ml.) was then added and the methylglucosides were hydrolyzed at 100° C. for 3 hours. Chloride ion was removed with silver carbonate and the silver ion with hydrogen sulfide. The solvent was removed under reduced pressure to yield a syrup, which was then examined on the paper chromatogram.

Enzymatic Methods

1) Preparation of the crude enzyme solution.—Soybean β-amylase was obtained by repeated extraction with water of finely ground soybeans, previously extracted with ether. The aqueous solution was centrifuged at 20,000 r.p.m. The crude enzyme was precipitated from the supernatant by slowly adding acetone. The fraction precipitating between 50 and 65 percent acetone was collected and dissolved in 5 ml. of distilled water. Enzyme activity was estimated according to the method of Noelting and Bernfeld (*10*): 1 ml. of soybean extract (1:10) liberated 2.8 mg. of maltose from soluble starch in 1 minute at 20° C.

2) Enzymatic degradation.—Fifty mg. of Fraction I were dissolved in 31 ml. of water, and 3 ml. of acetate buffer (10.2 ml. of acetic acid and 30.7 gm. of crystalline sodium acetate dissolved in 250 ml. of water, *p*H 4.8) were added. Six ml. of soybean β-amylase solution were added at once. Immediately after the addition of the enzyme solution, an aliquot of 1 ml. was withdrawn and the enzyme activity was destroyed by adding 2 ml. of alkaline 3,5-dinitrosalicylic acid reagent (*10*). This served as a blank for the estimation of the reducing sugars.

3) Reducing power.—The enzymatic degradation was followed by the estimation of the liberated reducing sugars, using 3,5-dinitrosalicylic acid reagent (*10*). At intervals, 1 ml. of the enzyme digest was withdrawn, and 2 ml. of dinitrosalicylic acid reagent were added. The mixture was then heated for 5 minutes on a boiling water bath. After rapid cooling in running water, the solution was diluted with 20 ml. of water and the extinction was measured in a Pulfrich photometer, using green filter S53. Maltose hydrate concentrations between 0.2 and 2.0 mg. per ml. were used as standards.

Periodate oxidation of the carbohydrate moiety.—Fraction IA (59.2 mg.) was dissolved in 55 ml. of 0.0436 *M* sodium metaperiodate. The solution had a *p*H of 4.5 and was kept at 0° C. At intervals, 5-ml. aliquots were withdrawn and added to a solution of 10 ml. of 0.05 *N* sodium arsenite, 1.5 gm. of sodium bicarbonate, and 3 ml. of *M* potassium iodide. After 15 minutes the excess of arsenite was titrated back with 0.05 *N* iodine

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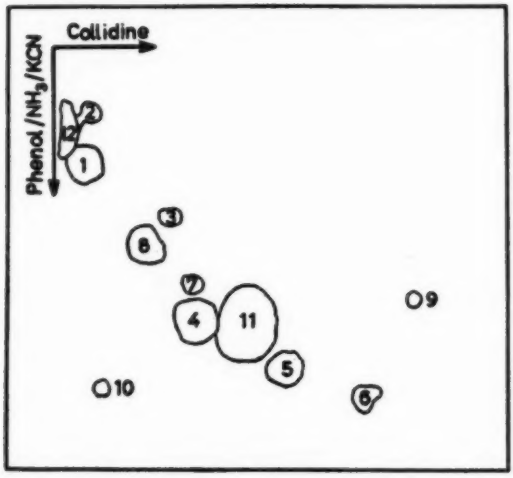
solution, using starch as an indicator. Another 5-ml. aliquot was added to 0.3 ml. of ethylene glycol, and the formic acid present was estimated after 20 minutes with 0.01 *N* sodium hydroxide using methyl red as an indicator. The results of this periodate oxidation are given in text-figure 3.

Hemorrhage-inducing activity.—Bioassays of a number of fractions have been performed by Shear and Perrault (*11*). The tumor-necrotizing activities they obtained will be briefly mentioned in these reports as personal communications. A more detailed account is expected from Dr. Shear. The most significant finding in this connection, however, appears to be that large amounts of reducing sugar can be eliminated from the P-25 starting material concurrently with a marked increase in biological potency.

Results

Identification of the amino acids.—The two-dimensional chromatogram on the hydrolysate of Fraction I showed twelve spots after developing with ninhydrin (text-fig. 1). Ten of the spots corresponded closely in position to those given by the following amino acids: glutamic acid, aspartic acid, serine, alanine, valine, leucine and isoleucine, threonine, glycine, tyrosine, and lysine. Two relatively large spots appeared that were not normally found in protein hydrolysates; one of them could be identified as glucosamine by treatment with acetylacetone and Ehrlich reagents as described by Partridge (*5*). In addition, a relatively large spot was detected, intermingled with the aspartic acid and glutamic acid



TEXT-FIGURE 1.—Two-dimensional chromatogram of the products of Shear's polysaccharide (Fraction I) obtained by hydrolysis with 6 *N* hydrochloric acid for 19 hours; developed in collidine followed by phenol-ammonia (2 percent). 1) Glutamic acid, 2) aspartic acid, 3) serine, 4) alanine, 5) valine, 6) leucine and isoleucine, 7) threonine, 8) glycine, 9) tyrosine, 10) lysine, 11) glucosamine, 12) α,ε-diaminopimelic acid (?).

spots, and having an unusually low R_F value in collidine and phenol. The position of this spot would suggest the presence of α,ϵ-diaminopimelic acid (DAP), which is frequently found in microorganisms (*12*). Further evidence may confirm the presence of DAP. Alanine, valine, glycine, and glutamic acid predominate; sulfur-containing amino acids were absent or present in very small amounts.

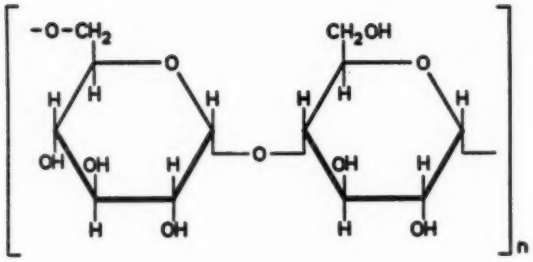
Qualitative and quantitative estimation of sugars and hexosamines.—The products of complete hydrolysis of Fraction I with 1.5 *N* hydrochloric acid (19 hours, 100° C.), when examined by paper chromatography, gave rise to three spots after developing with aniline-oxalate reagent. Two spots corresponded very closely in position with glucose and glucosamine, whereas the third spot showed an R_F value somewhat lower than that of hexuronic acids. Glucosamine was definitely identified by spraying with alkaline acetylacetone followed by *p*-dimethylaminobenzaldehyde reagent (*5*). The glucose spot appeared at first to be a multiple spot. Chromatograms carried out with varying solvent systems and varying amounts of hydrolysate, however, excluded the presence of another aldohexose.

The quantitative estimation of the sugars on the paper chromatogram indicates that of the total reducing power of 77 percent, 52 to 55 percent was accounted for by glucose, 12 to 15 percent by glucosamine, and 6 to 9 percent by the material of the third spot. The extract from the third spot gave a weakly positive test for hexuronic acid with Dische's carbazol reagent (*8*); less than 2 percent of hexuronic acid was found.

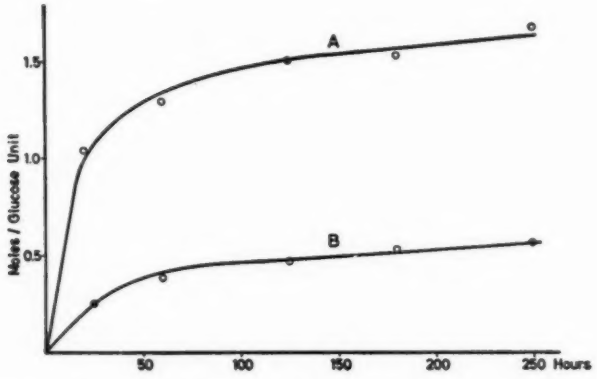
The chemical constitution of the glucan.—Paper chromatography of the methylated sugars, obtained by methanolysis and hydrolysis of the methylated Fraction IA, gave rise to two spots after developing the chromatogram with ammoniacal silver nitrate. The two spots were of approximately the same size and intensity, and were identified as 2,3,4-trimethylglucose and 2,3,6-trimethylglucose by comparison with concurrently run reference samples. Chromatograms run with greatly increased concentrations of the hydrolysate led to the detection of two other spots corresponding to 2,3,4,6-tetramethylglucose and an unidentified sugar. The presence of equal amounts of 2,3,4- and 2,3,6-trimethylglucose and an almost negligible amount of tetramethylglucose would indicate that the glucose units are linked together in an unbranched or slightly branched chain through alternate 1,4- and 1,6-linkages. The repeating unit of the glucan would therefore be a disaccharide, for which the configurations of the glucosidic linkages are, however, not unequivocally established. The high positive optical rotation ($[\alpha]_D^{20} = +193°$ in water; Fraction IA) and the downward drift in rotation observed during hydrolysis suggest the α-type linkage. The structure of the glucan is tentatively represented by the repeating unit shown in text-figure 2.

This structure is corroborated by periodate oxidation, since the repeating disaccharide unit, in fact, consumes approximately 3 moles of periodate with the production of 1 mole of formic acid (text-fig. 3).

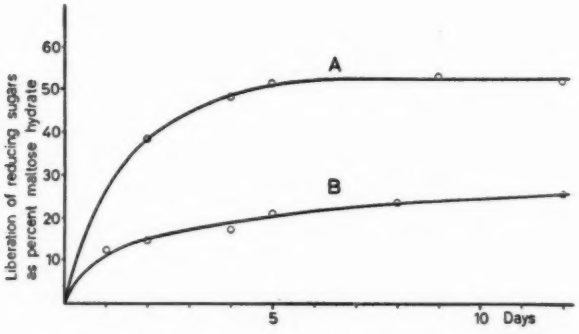
Enzymatic treatment of the active Fraction I with a crude soybean β-amylase preparation and subsequent bioassay (*11*) of the remaining ma-



TEXT-FIGURE 2.—The repeating unit of the glucan.



TEXT-FIGURE 3.—Oxidation of Fraction IA from Shear's polysaccharide with sodium metaperiodate at *p*H 4.5. Temp.=0° C. *Curve A:* Periodate consumption, expressed in moles periodate reduced by 1 mole glucose unit. *Curve B:* Production of formic acid, expressed in moles per mole glucose unit.



TEXT-FIGURE 4.—Enzymatic degradation of Fraction I with crude (*Curve A*) and purified soybean β-amylase (*Curve B*) at *p*H 4.8.

terial indicates that the glucan can be largely removed without loss of activity. The optimum liberation of reducing sugars was reached after 12 days at 25° C. and represents 53 percent of the dry weight, expressed as maltose hydrate (text-fig. 4). Glucosamine estimations performed on the enzyme digest after prolonged dialysis showed that the glucosamine content was unchanged. With highly purified soybean β-amylase preparations, degradation limits of only 20 to 33 percent (expressed as maltose hydrate) were obtained (text-fig. 4). Bioassays performed by Shear and Perrault (*11*) indicate that Fraction I, after digestion with a crude soybean β-amylase, had a somewhat higher hemorrhage-inducing activity than the P-25 starting material. Blank assays on equivalent amounts of the enzyme used showed no effect.

Discussion

In this and the preceding paper (*1*), evidence is presented that the tumor-hemorrhagic material P-25 yields two polysaccharides: a glucan, and another polysaccharide containing N-acetylglucosamine as a main constituent. This is now supported by the fact that the glucan can largely be removed from the active complex without loss of potency by enzymatic degradation with crude soybean β-amylase, whereas the N-acetylglucosamine-containing polysaccharide remains unaffected. The degradation limit of 53 percent, expressed in maltose hydrate, as compared with about 60 percent of glucose present in the active complex, suggests that the main product of hydrolysis is a disaccharide. With highly purified soybean β-amylase preparations, degradation limits of only 20 to 33 percent (expressed as maltose hydrate) were obtained. It is therefore reasonable to assume that the splitting of glucosidic bonds is not due, or is due only to a small extent, to β-amylase which splits 1,4-linkages only, but is possibly due to the presence of a Z-enzyme [Peat *et al.* (*13*)] which supplements the β-amylase action.

From our results it thus appears that most of the carbohydrate material of the active complex is not essential for its tumor-hemorrhagic activity. The smallest active unit obtained to date still contains phospholipid, protein, and carbohydrate firmly linked together. This carbohydrate contains all the N-acetylglucosamine and a small amount of glucose. Splitting of either of these components by physicochemical or chemical means has so far invariably led to loss of potency. Removal of the bound phospholipid (*14*), and also the separation of the carbohydrate moiety with picric acid, resulted in the complete loss of potency of all components. These facts strongly suggest that the tumor-hemorrhagic activity may depend upon the intact structure of the complex [*cf.* Hartwell *et al.* (*14*) and also Zahl (*15*)].

Physicochemical data obtained by Malmgren (*2*) show furthermore that the tumor-hemorrhagic activity need not be restricted to a particular particle weight, since bacterial preparations with varying degrees of aggregation show similar degrees of potency. The appreciably higher electrophoretic mobility of our preparation as compared with that investi-

gated by Kahler, Shear, and Hartwell (*3*) would, however, suggest a significant difference in chemical composition or a different arrangement of the components in the complex, resulting in a higher electric charge density. The nature of this discrepancy is not as yet understood since the differences in chemical composition are too small to account for the higher electrophoretic mobility.

Summary

Further evidence is presented that the carbohydrate part of the tumor-hemorrhagic agent of *Serratia marcescens* consists of a glucan and of a polysaccharide containing N-acetylglucosamine as a main constituent. The glucan could largely be removed from the active complex by digestion with crude soybean β-amylase. This procedure led to an increase in potency; the glucan may therefore be considered as an inactive storage material built up from the excess of glucose present in the culture medium. The glucan is built up of glucose residues probably linked together in an unbranched or slightly branched chain by alternate 1,4- and 1,6-glucosidic bonds. The protein moiety contains at least ten amino acids; alanine, valine, glycine, and glutamic acid predominate. The smallest active unit obtained to date still contains phospholipid, carbohydrate, and protein firmly linked together in a complex. It is suggested that the intact structure of the latter lipid-polysaccharide-protein complex is essential for the tumor-hemorrhagic activity.

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Physicochemical Characteristics of the Tumor-Necrotizing Agent from *Serratia marcescens* (Shear's Polysaccharide)[1,2]

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In 1943 Kahler, Shear, and Hartwell (*1*) investigated the trypsin-treated fractions of the polysaccharide from culture filtrates of *Serratia marcescens*, which produces hemorrhagic necrosis in tumors. These preparations, labeled K-15c, P-2, and P-3, were characterized as to homogeneity and molecular weight by means of ultracentrifugation, diffusion, and electrophoresis. The present author has applied the same methods to a more recent preparation called Lot No. P-25,[3] the chemical properties of which are described by Rathgeb and Sylvén (*2*, *3*).

Experimental

Diffusion

The diffusion constant was determined according to the scale method of Lamm (*4*), at 20° C. and *p*H 6.8 in a 0.1 μ phosphate buffer + 0.1 *M* NaCl. The diffusion constant of P-25 calculated according to the moment method was found to be 2.15×10^{-7} c.g.s., and calculated from the area of the diffusion curves it was found to be 1.80×10^{-7} c.g.s. This discrepancy indicates a nonhomogeneous material.

Sedimentation

The sedimentation of P-25 in the ultracentrifuge was studied according to the technique of Svedberg and Pedersen (*5*), using a centrifugal force of 200,000 $\times$ g and a 0.1 μ phosphate buffer at *p*H 6.8 + 0.1 *M* NaCl. The centrifuge diagrams in all runs revealed a heterogeneous material (text-fig. 1). The main constituent had a sedimentation constant, *s*, of about 5 S (Svedberg units); another constituent of lower concentration had an *s* value of 12 S.

Electrophoresis

The electrophoretic mobility of P-25 was recorded by the moving boundary method of Tiselius (*6*) and Svensson (*7*). The experiments

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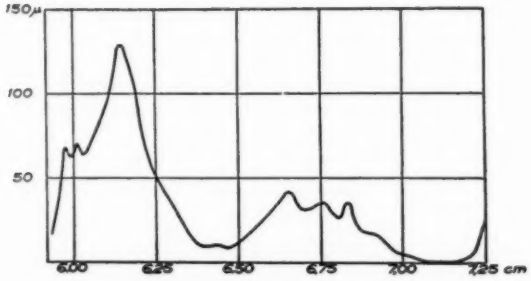
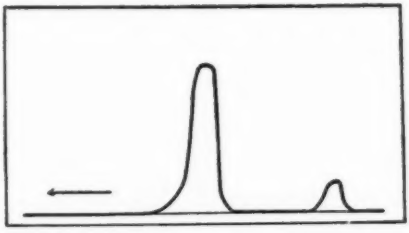
[3] Preparations kindly supplied by Dr. M. J. Shear.



TEXT-FIGURE 1.—Sedimentation diagram of P-25 after 30 minutes at 200,000 × g. *Ordinate:* scale line displacement; *abscissa:* distance from axis of rotation.

were performed at +0.5° C. in 0.1 μ buffers. The electrophoretic patterns exhibited only one component moving toward the anode with a mobility of 10×10^{-5} cm.^{2} per volt second (text-fig. 2). In one experiment at *p*H 6.8, the solution was taken out from the five different parts of the cell after the run, the buffer salts were dialyzed off, and the remaining polysaccharide material was dried *in vacuo* in the frozen state. However, the biological potency per milligram of substance and also the chemical composition, so far as hexosamine, Kjeldahl nitrogen, and total carbohydrate content were concerned, were almost the same for all five fractions. Hence, it seems impossible to purify the active material further by electrophoresis.



TEXT-FIGURE 2.—Electrophoretic pattern of P-25 after a 57-minute run at *p*H 6.8. Direction of migration indicated by the arrow. The small peak is a false boundary.

Molecular Weight

Evidently it is meaningless to calculate the molecular weight of such a heterogeneous material as P-25. Only a very rough estimation of the order of magnitude is possible. The partial specific volume, *V*, was found to be 0.75 (*1*). Adopting this value, a particle weight of the order of magnitude of some hundred thousands can be calculated from Svedberg's formula.

The physicochemical constants of P-25 are compiled in table 1; previous data by Kahler and co-workers (*1*) on polysaccharides prepared from culture filtrates are included for comparison.

TABLE 1.—*Physicochemical constants of polysaccharide preparations*

Preparation	Concentration (*percent*)	Buffer solutions	pH	Mobility $\times 10^5$ cm.^{2}/volt sec.	Sedimentation constants of main components $\times 10^{13}$ c.g.s.	Diffusion constant $\times 10^7$ c.g.s.	Molecular weight
P-25	0. 50	Phosphate..... 0. 1 μ + 0. 1 *M* NaCl	6. 8	.....	4. 6 and 12. 4	.....	Some hundred thousands
	. 25	Phosphate..... 0. 1 μ + 0. 1 *M* NaCl	6. 8	9. 5	5. 3 and 10. 9	1. 8	
	. 25	Barbiturate..... 0. 1 μ	8. 6	10. 5			
	. 25	Acetate..... 0. 1 μ	4. 8	9. 7			
Rest P-25	. 4	Phosphate..... 0.1 μ + 0.1 *M* NaCl	6. 8	.....	(See text)	.....	Below 15, 000
P-2	. 26	Acetate..... 0. 1 μ +0. 2 *M* NaCl	4. 1	.....	70. 6	0. 89	8×10^4
		Acetate..... 0. 1 μ	4. 1	2. 59			
P-3	. 5	Phosphate..... 0. 1 μ	7. 4	2. 47	234		

After β-amylase treatment the remaining material, called "Rest P-25" (*3*), was found to be of lower particle size. The ultracentrifuge curve presented a material that even after 85 minutes at 200,000 $\times$ g had not left the meniscus. Hence the molecular weight is estimated to be of the order of about 15,000 or less.

The preparations show large variations in molecular weight. The new P-25 preparation has a considerably smaller molecular weight than the previous preparations, P-2 and P-3. It is, however, likely that the smallest active unit is associated with the material called "Rest P-25" (*3*).

The electrophoretic mobility of about 10×10^{-5} indicates a high electronegative charge, which cannot be accounted for by the components found by Rathgeb and Sylvén (*2*, *3*), who did not find ester sulfate, hexuronic acids, or other strongly charged groups in appreciable amounts. Some other highly charged material, not yet demonstrated, must therefore be assumed to be present in P-25. This material may be a dissociating phosphate group since P-25 contains about 1 percent phosphorus.

Summary

The preparation P-25 was polydisperse, and the estimated molecular weight was of the order of some hundred thousands, a much lower value than those found in previous preparations. The electrophoretic mobility was found to be 10×10^{-5} cm.^{2} per volt second, which is higher than can be expected from the presently known components.

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Alkaline Phosphatase Activity During Carcinogenesis of Mammary Tumors in Mice Implanted With Stilbestrol Pellets [1,2]

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Many papers have been published on the stages of development of mammary tumors in mice, following the work of Lacassagne (*1*) who in 1932 induced mammary carcinomas in mice by weekly injections of crystalline benzoate of estrin. Thus in 1935 Burrows (*2*) studied the progressive changes in the mammary glands of 199 mice, 130 of which were treated with estrogen or allied compounds. Progressive dilatation of ducts containing pink homogeneous material was seen, particularly in the treated animals. This was also noted in 1940 by Taylor and Waltman (*3*), who treated C57 and DBA mice with estradiol benzoate and estrone, which caused moderate proliferation of acini in the mammary glands of the C57 mice and intensification of the inherent tendency to acinar formation in the DBA mice. The most striking change found in the mice receiving estradiol benzoate for long periods of time was the retention of secretions in the acini and ducts. Bonser (*4*) in 1945 studied mammary carcinomas occurring spontaneously in RIII and "White Label" females, as well as those induced with estrogens in males of the same strains, and found cystic distentions and secretion in the mammary gland acini. The acinus was thought to be the predominant site of cancer formation, intraductal carcinoma with papillary formation being very rare. It was thought that the effect of the milk factor was to produce a high degree of acinar proliferation, and intra-acinar and extra-acinar carcinoma. Huseby and Bittner (*5*), however, in their studies on a large number of untreated animals, concluded that the milk factor did not alter the extent to which lateral buds occurred along the larger ducts of the mammary gland.

Cytochemical or histochemical studies of enzymes in mammary glands during growth and carcinogenesis, either of spontaneous or estrogen-induced mammary tumors, have not come to our attention although these disciplines were applied to the mammary gland during various

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states of activity by Dempsey *et al.* (*6*) in 1947. Using several methods they particularly studied the alkaline phosphatases, utilizing as substrates glycerophosphate, nucleic acid, diphosphate, and glucose 1-phosphate, adjusted to *p*H 9.4, with incubation times of 3, 6, and 24 hours. Alkaline phosphatase was found to occur prominently in the mammary gland and the localization was similar in several species examined. They described certain epithelial "basket cells" in the basal-cell layers of ducts and alveoli which they thought were myoepithelial in origin. These cells were first described in 1894 by Benda, who considered that they had contractile functions. They reacted very strongly for alkaline phosphatase, were devoid of sudanophilia, and were eosinophilic. Certain endothelial cells contiguous to the basement membrane also showed phosphatase reaction, as did capillaries. During pregnancy, and especially during lactation, when the alveoli were distended, these basket cells did not multiply in proportion to the enlarged alveoli. They lost their former syncytial character and became fenestrated. The capillaries increased and came in contact with the basement membrane, with an increase in alkaline phosphatase activity. With the incubation times used, the cytoplasm of cells in the ducts, alveoli, and the content of the alveolar lumen reacted positively. The material in the lumen was particularly reactive for phosphatase when nucleic acid was used as a substrate. It was suggested by Dempsey and coworkers that the marked activity of alkaline phosphatase in these "basket cells," together with their close proximity to the lining of ducts and alveoli, might indicate that they served as transmitters of materials across membranes for synthesis and secretion.

It has been shown by Pearson *et al.* (*7*, *8*), in tumors with reportedly high alkaline phosphatase activity such as liver tumors induced in rats by azo dyes, that this high activity was mainly due to proliferating bile ducts and vascular sprouts rather than to the activity of the tumor cells *per se*. In a subsequent study of alkaline phosphatase activity in four transplantable tumors of the breast, marked differences were observed by Pearson and Richardson (*9*). In one of these tumors, the H2712, a luminal distribution of activity was strikingly apparent. In two other tumors, E0771 and dbrB, phosphatase-positive material was present in the small lumina as well as in granules within the cells. This became apparent only when short incubation times were used, and suggested that secretory phenomena had persisted in the tumor cells.

Because of the ancillary role of the enzyme in liver tumors and the secretory phenomena suggested by the previous study on mammary tumors, as well as the lack of such information concerning the progressive stages of carcinogenesis in mammary tumors in mice, it was felt that the following study would be of value.

Methods and Materials

One hundred sixty mice were used for this experiment, 87 males and 73 females. They were F_1 hybrids between RIII females and C57L males.

The RIII strain has a high incidence of mammary tumors in the breeding females and a low incidence in virgins. The C57L is a low-mammary-tumor strain.

These (RIII $\times$ C57L)F_1 hybrids were used since Richardson (*10*) has shown that the mammary glands were better developed in males of this type of hybrid than in males of most strains. The third pair of glands, which was used for histochemical and histological studies in this experiment, was present in nearly 100 percent of the animals. Furthermore, these mice tolerated treatment with stilbestrol better than many other strains did.

Solid pellets of stilbestrol in cholesterol were prepared by a modification of the technique described by Shimkin and White (*11*), and were implanted subcutaneously by trocar. Each pellet weighed 5 to 6 mg. and contained 20 percent stilbestrol and 80 percent cholesterol. Pellets were implanted into 8-week-old animals, which were regularly sacrificed at 5, 6, 7, 8, and 9 months of age, and also when palpable tumors appeared.

The animals were killed by ether. The skins were removed leaving the mammary glands adherent. One of the third pair of glands was immediately removed and flattened by placing it on a piece of paper, and both gland and paper were placed in ice-cold acetone for 24 hours. The gland was then placed in cold absolute alcohol overnight, followed by two changes of benzene for forty-five minutes each. In animals having palpable tumors, a thin slice of the tumor was taken and treated in the same manner. The tissues were then placed in paraffin at 53° to 55° C. for 2 hours (half of this time in the vacuum oven) for infiltration with paraffin and were then embedded in paraffin. The slides were run through xylol, absolute alcohol, 95 percent alcohol, and water, and then incubated at 37° C. in a substrate composed of 2% sodium barbital, 2% sodium β-glycerophosphate, distilled water, 2% calcium chloride, 2% magnesium sulfate, and adjusted to *p*H 9.4. They were then rinsed in 1% calcium chloride and placed in 2% cobalt nitrate, distilled water, and dilute ammonium sulfide. Incubation times in the substrate were 1 minute, 5 minutes, 15 minutes, and 1 hour. Controls were run by omitting the sodium β-glycerophosphate of the above substrate solution and incubating at 5 minutes and 1 hour. The control sections were all negative. All the tissues were also stained with hematoxylin and eosin.

To relate the histochemical observations to the histogenesis of the tumor, simultaneous sections from the same block were stained routinely with hematoxylin and eosin.

In order to study the whole gland, the skins with the remainder of the mammary glands attached were pinned out flat in a pan containing paraffin, covered with Dubrasque-Brasie fixative [3] for 12 to 24 hours, and then stored in 80 percent alcohol. Whole mounts were made of the third gland. These were stained with Meyer's hematoxylin and studied under the dissecting microscope.

[3] Picric acid, 16 grams; normal saline, 1,000 cc.; 95 percent alcohol, 1,600 cc.; glacial acetic acid, 160 cc.; formalin, 320 cc.

Observations

The mammary glands of animals sacrificed at five months of age showed the large ducts lined with one layer of cuboidal epithelium. These ducts were surrounded by one or two layers of connective tissue. Within each duct there was material that stained pink with hematoxylin and eosin, and in many instances was organized into concretions. There was also a mild proliferation of acini. Two tumors were palpable during the fifth month. In the six-month-old mice there were no essential differences to be observed. Hyperplastic nodules were seen in a number of instances, either in the whole mount or the sections.

In the mice killed at seven months of age there were striking differences. Many more hyperplastic nodules could be seen. Some of the medium-sized ducts were now lined with two or three layers of cells and were proliferative. In many areas of acinar proliferation and hyperplastic nodules there was also an increase in fibrous tissue. Concretions in the ducts were very prominent. During the eighth and ninth months these changes were accentuated; there was an increase in the size and number of the hyperplastic nodules and a progressive dilatation of the ducts, with multiple concretions. This was especially apparent in studying the whole mount.

The tumors that appeared were adenocarcinomas, mainly of two general types: a relatively compact tumor with numerous small acini of rather uniform size, and a subtype in which the acini were larger and showed considerable variation in size. The former presumably arose from hyperplastic areas or clusters of small acini. The latter type, which was more frequent, appeared to arise from large acini that occurred in aberrant areas of hyperplasia, or from the walls of proliferating ducts. In these latter tumors, which were often mixed with the former, there was a great deal of papillary infolding of the epithelium which at times filled the ducts and/or acini and caused the appearance of irregular slit-like lumina. Small lumina, similar to those in the first type of tumor, could be seen in the more compact intraluminal papillary infoldings. Some of these tumors had a great deal of fibrous tissue present which distorted their appearance. During the sequence of stages of tumor formation, no essential differences could be distinguished between the male and female animals.

The histochemical data on alkaline phosphatase was of great interest. Animals sacrificed at five and six months of age showed rather intense reactions in the large and medium-sized ducts. The first reaction apparent at incubation times of 1 and 5 minutes was in the external layer of the ducts, in certain cells that correspond in location to the myoepithelial cells (fig. 1). These cells also showed strong activity in the hyperplastic nodules and proliferative acini (figs. 3, 4). In addition, the luminal border in certain areas showed some reaction. Many round, intensely reactive granules of varying sizes were seen in the cytoplasm of the epithelial cells in many of the proliferating acini (fig. 2).

In mice seven, eight, and nine months old, when there was an increase of connective tissue in the hyperplastic nodules the concretions were more

numerous and striking. They reacted more intensely for enzyme and showed greater tendency toward ring formation due to stasis of secretions (figs. 5, 6). The increased connective tissue was negative for alkaline phosphatase. In areas where the hyperplastic nodules were composed of epithelium consisting of a single layer of cells and were otherwise similar to those found in 5- or 6-month-old mice, the myoepithelial cells showed strong reaction. However, one change was striking—a progressive tendency toward an increase in the layers of the luminal epithelial cells. This luminal epithelium became two or three layers thick, and the cells were large and had assumed a distinct appearance of proliferating. In these proliferative acini a thin margin of the luminal cells reacted for enzyme, and the reaction was apparent after incubation for five minutes (fig. 6). The myoepithelial cells revealed a complete reaction only where the external surfaces of these acini were in proximity.

In certain areas where early tumor formation could be demonstrated and ascribed directly to this proliferating epithelium of the ducts and acini, the epithelium had many layers of cells and the lumina were of various sizes, often small. These tumors under these conditions gave intense reaction for enzyme at their luminal surface. The other cells did not react and no myoepithelial cells were apparent (figs. 7, 8).

Generally in tumors of the first type, composed of acini with small lumina (fig. 9), only a thin margin of the luminal cells showed activity. The activity was apparent after incubation for one minute and became more intense with longer incubation. Alkaline phosphatase-positive material very often filled these lumina and reacted very intensely (figs. 10, 12). In tumors with large lumina the internal border also reacted. In many tumors, in addition to areas containing large lumina, there were compact areas nearly devoid of such structures, and in such areas there was very little reaction for the enzyme (figs. 11, 12). In many instances the basal or myoepithelial layer reacted, particularly where the external surfaces of acini were in approximation. Here in some instances the reaction gave an impression of endothelial-lined spaces. In the tumor that could be regarded as an intrapapillary carcinoma, only the lining of lumina showed activity. Where small acini appeared within the papillary projections these also showed reaction. The connective tissue did not react. In all types of tumors granules could be seen, but they were not as marked as in the proliferative acini and hyperplastic nodules previously described.

Discussion

It has been observed in mammary tumors in mice and human endometrial carcinomas that there was a change or "loss" of alkaline phosphatase activity in rapidly growing tumors or in areas in which the tumor is especially anaplastic (*12–14*). This may be due to several factors that are not entirely understood. Differences between tumors certainly exist in regard to their alkaline phosphatase activity. In a fully developed spontaneous or transplanted mammary tumor, especially when long incubation times of one or more hours are used, enzyme activity may be slight or

absent in its peripheral or central areas. If such a fully developed tumor is examined with a single long incubation time, one gets a strong impression that there is a change or loss of activity incident to anaplasia.

In our examination of the progressive stages of development, where the tumor was considered to be derived from transformations of the normal growth structures such as ducts and acini, there seemed to be no variation in enzyme intensity from the nontumorous areas of estrogen-stimulated breast to the hyperplastic nodules and finally to the fully developed tumor. There was only a rearrangement of cells and structures, which remained normally active. It is possible that if more tumors could be studied in this manner, similar results could be obtained.

Before the tumor was formed, the "myoepithelial" cells and endothelial-lined spaces reacted intensely in cases where ducts and acini were in juxtaposition (fig. 4). These phenomena were duplicated in certain of the fully developed tumors, especially in those composed of large acini in close proximity to one another, where there were reaction centers corresponding to the location of the myoepithelial cells. Also present were endothelial-lined clefts which showed prominent enzyme reaction. In the more complex intrapapillary tumors, these clefts were also evident. Casts and concretions which reacted positively were also present in the intrapapillary tumors and were identical with those seen in the dilated ducts. In the more complex tumors, there was positive reaction in the luminal lining, in a narrow portion of the bordering cytoplasm, similar to the reaction of the brush border of the convoluted tubules in the kidney. In the tumors with small acini, alkaline phosphatase-positive material was present in the lumina and the extreme luminal surfaces showed intense reaction. The enzyme activity of these small alveolar tumors was very high because of a larger luminal surface area. There was no reaction on the external surfaces except where acini were large and were in proximity to each other. In many areas granules appeared in the cytoplasm.

It would seem, therefore, that the intensity of the alkaline phosphatase activity did not change, under the conditions of this experiment, during the induction of tumors by estrogen. Several observers have noted that luminal concretions are more frequent in estrogen-induced tumors than in spontaneous tumors. Whether or not prolactin in addition to estrogen would increase this propensity is an interesting question. The examination at intervals of animals developing spontaneous tumors would also be of interest, and if compared with animals developing estrogen-induced tumors might reveal an effect of the estrogen which might conceivably play a part in the early phases of tumor formation.

We believe, therefore, that in these experiments with estrogen-induced proliferation and tumor formation, the essential reactions at the luminal borders, the secretory granules, the alkaline phosphatase-positive concretions and myoepithelial cells, and the endothelial-lined spaces are all expressions of membrane phenomena where exchange and synthesis of materials for secretion takes place in a gland that originally possessed these functional properties. In the tumor, particularly when induced by

estrogen, this physiological function is present in the growing acini, proliferating acini, hyperplastic nodules, and in the tumor. The final configuration of the tumor eventually determines the sites of these activities. In tumors where the surface area is large (very small acini), the total activity is increased. A tumor that has been transplanted for many generations tends to have a certain stable pattern and the enzyme variation between individual tumors is small. A spontaneous tumor or a tumor induced by estrogen may vary in structure and differences in the total enzyme activity may be very great.

Summary

(1) One hundred sixty F_1 hybrids of RIII females and C57L males were implanted with estrogen pellets at 8 weeks of age and sacrificed at 5, 6, 7, 8, and 9 months of age, and also when palpable tumors appeared.

(2) Histologic descriptions of the stages of tumor development are given, and correlated with the histochemical reactions for alkaline phosphatase.

(3) The most striking phenomenon was the lack of basic differences in the intensity of reaction during the various stages of tumor formation, although shifting changes in distribution were seen, due to the growth and architecture of the tumor.

(4) Areas that reacted for alkaline phosphatase seemed to be those concerned with the transport of materials for synthesis and secretion.

(5) Alkaline phosphatase activity under the conditions of this experiment was not dependent upon the malignancy or anaplasia of the tumor, but upon the activity exhibited by the luminal borders, myoepithelial cells, and endothelial-lined spaces.

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PLATE 82

Breast changes in (RIII × C57L)F_1 hybrids, treated with stilbestrol. Alkaline phosphatase reaction after incubation in substrate for one hour. No counterstain used.

FIGURE 1.—Breast of mouse #2, female, sacrificed at 5 months of age. There is alkaline phosphatase-positive material in the lumen of a large duct, and there are concretions in small proliferating acini. These acini show intense enzyme reaction in the "basket" or myoepithelial cells. × 120

FIGURE 2.—Breast of same mouse as shown in figure 1, under higher magnification. Numerous discrete granules of varying sizes are seen in the cytoplasm of the epithelial cells. Many coalesce to form larger masses. Alkaline phosphatase-positive material is seen in the lumen, directly associated with the granules. × 500

FIGURE 3.—High-power view of a hyperplastic nodule in the breast of mouse #57, female, sacrificed at 6 months of age. Intense alkaline phosphatase reaction in "basket" or myoepithelial cells. × 500

FIGURE 4.—High-power view of a hyperplastic nodule with large acini in the breast of mouse #38, female, sacrificed at 6 months of age. Intense enzyme reaction in "basket" or myoepithelial cells where walls of acini are in juxtaposition. Remaining cells give faint reaction. Unorganized alkaline phosphatase-positive material is present in the lumen. × 500

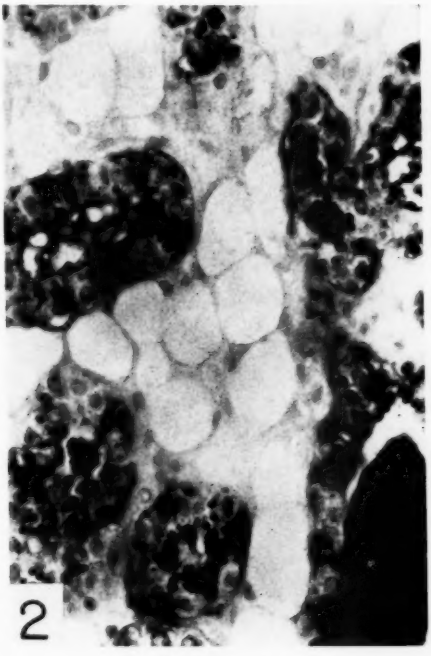

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PLATE 83

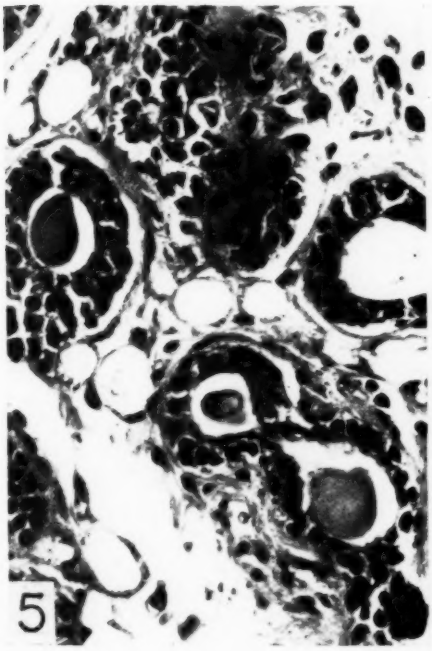
Breast changes in (RIII × C57L)F_1 hybrids treated with stilbestrol. Alkaline phosphatase reaction after incubation in substrate for one hour.

FIGURE 5.—Breast of mouse #84, female, sacrificed at 7 months of age. Section shows high-power view of a fibrotic hyperplastic nodule. Epithelial cells of the acini have proliferated to form several layers. Cells and nuclei of acini have become larger. Hematoxylin and eosin. × 500

FIGURE 6.—Breast of same mouse as shown in figure 5. Note intense alkaline phosphatase reaction of extreme luminal borders of the cells. Remaining portions of the epithelial cells react very faintly. Concretions with positive enzyme reaction may be seen. A portion of the basal cells and endothelial-lined space are shown in acinus in lower right. × 500

FIGURE 7.—Breast of mouse #99, female. Sacrificed at 8 months of age. Section shows a high-power view of a very early intraluminal carcinoma. Hematoxylin and eosin. × 500

FIGURE 8.—Breast of the same mouse as shown in figure 7. The acini are the same ones seen in figure 7. Phosphatase reaction is confined to a very narrow margin of the cells of the luminal surface. Positive material is present in lumen. × 500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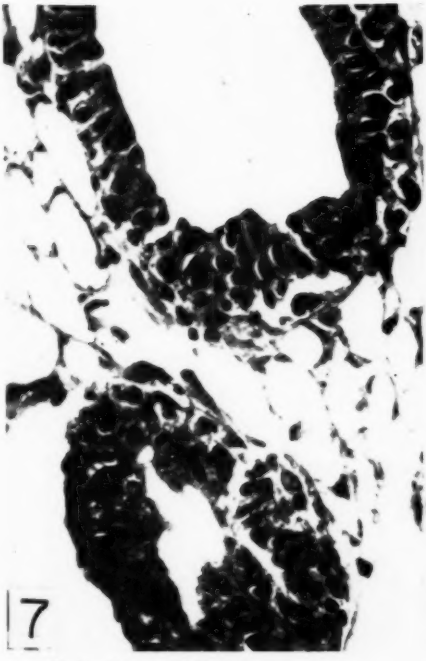
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PLATE 84

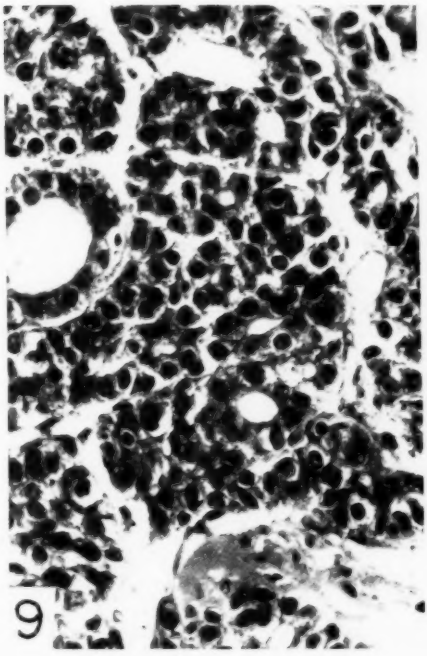
Breast changes in (RIII × C57L)F_1 hybrids treated with stilbestrol.

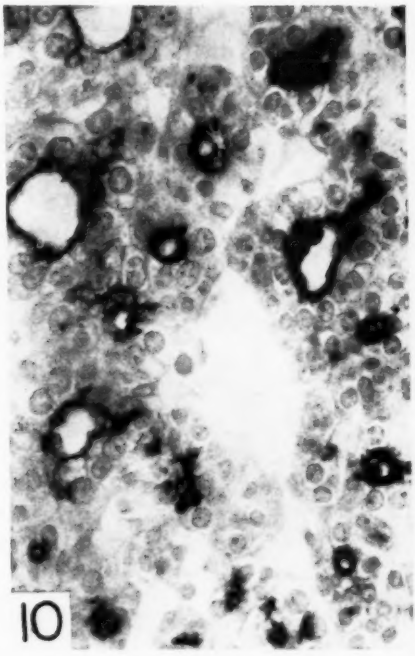
FIGURE 9.—Breast of mouse #112, female. This mouse had a palpable tumor and was sacrificed at 8 months of age. Section shows several small acini with small lumina. Some larger acini are seen. Hematoxylin and eosin. × 500

FIGURE 10.—Tumor of the breast of the same mouse as shown in figure 9. Alkaline phosphatase reaction is seen in the extreme luminal borders. Some very small lumina are filled with enzyme-positive material. Small granules can be seen in the cytoplasm of some cells. The remaining portion of the cells react faintly. Incubation time in substrate was 5 minutes. × 500

FIGURE 11.—Breast of mouse #101, female. This mouse had a palpable tumor and was sacrificed at 8 months of age. The upper half of the section shows tumor composed of large and small acini, while the lower half is solid and devoid of acini. Hematoxylin and eosin. × 120

FIGURE 12.—Tumor of breast of same mouse as shown in figure 11. The upper half of section shows intense alkaline phosphatase reaction at the extreme luminal borders. The more solid portions of the tumor, nearly devoid of acini, show very slight reaction. Incubation time in substrate was 15 minutes. × 120





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Induction of Tumors in Mice Following Intrasplenic or Intrahepatic Transplantation of Fetal Skin, Muscle, or Stomach with Methylcholanthrene[1,2]

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Although tumors may be induced frequently in the mouse when skin, muscle, or lung is exposed to the carcinogenic hydrocarbons, hepatic and splenic tissues have generally been observed to be refractory to the same carcinogens (*1*). Tumors have been observed in both liver and spleen, however, following implantation of fetal skin and methylcholanthrene (*2*); in these experiments, the intrasplenic tumors included squamous-cell carcinomas as well as fibrosarcomas, whereas in the liver only squamous-cell carcinomas were observed. Also, the incidence of induced tumors was higher in the spleen than in the liver. Since a comparatively small number of animals had been employed, it was considered necessary to reinvestigate the problem using larger groups. In addition to fetal skin, fetal muscle tissue was employed from a strain highly susceptible to the induction of fibrosarcomas. Answers were sought to the following questions: 1) Was the spleen a more favorable site than the liver for tumor induction? 2) Could the liver or the spleen inhibit development of fibrosarcomas in susceptible tissues?

It has been observed that fetal skin grafts in the mouse encyst when transplanted intramuscularly (*2–4*) or to the liver or spleen (*2*). Since carcinogen administered with the grafts is frequently contained within these cysts, prolonged contact between implanted epithelium and carcinogen is assured. If encystment were to occur similarly with transplants of fetal stomach, it might afford a method for the production of gastric carcinomas. In view of the lack of sarcomas previously observed following intrahepatic transplantation of fetal skin and methylcholanthrene (*2*), it was expected that induction of carcinomas with fetal stomach transplants would be favored in the liver.

Materials and Methods

A total of 120 young adult male mice of strain C3H/Jax were employed. The animals were housed in an air-conditioned room (76° to 78° F.) and

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[2] Supported by grants from the Florida Division of the American Cancer Society and the Damon Runyon Memorial Fund, and by grant C-1709 of the National Cancer Institute, National Institutes of Health, U. S. Public Health Service.

[3] The technical assistance of Lois Sumner, Hilda Banks, and Virginia Paul is gratefully acknowledged.

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were provided with a continuous supply of Purina laboratory chow pellets. Tap water was available at all times.

Fetal transplants were prepared as follows: Pregnant C3H mice were sacrificed 19 days following detection of a vaginal plug, and the fetuses removed aseptically. Skin from each fetus was removed with fine forceps and iridectomy scissors and placed in a sterile petri dish. Portions of the body-wall musculature together with fore and hind limbs freed of skin were also excised and deposited in a second petri dish. The stomach was removed and cut on the glandular side on a line approximately parallel to the limiting ridge. The forestomach segment was discarded, and the remaining glandular portion placed in a third petri dish. The skin in petri dish No. 1 was minced with scissors until the resultant fragments approximated 1 to 2 mm. in diameter. A similar procedure was employed for petri dish No. 2 which contained muscle tissue predominantly. The glandular stomach transplants in petri dish No. 3 were not minced. At the time of transplantation, each graft was tamped lightly with crystals of methylcholanthrene (MCA). Wherever possible, an attempt was made to wrap fetal grafts around the carcinogen. In the case of stomach grafts, crystals of MCA were inserted directly into the glandular lumina. All tissues were transplanted using a No. 15G trocar, and each mouse received either two grafts of skin, two of stomach, or two of muscle, etc.—one graft being placed in the liver and the other in the spleen.

All mice were palpated for tumors weekly, beginning with the 4th month following the time of operation and continuing for the duration of the experiment. While many of the mice were observed for 300 or more days, especially those bearing stomach transplants, animals with large abdominal masses as well as those in apparent poor health were often sacrificed sooner. All tissues were fixed in Tellyesniczky's fluid, sectioned at 3 to 4 micra, and stained with hematoxylin and eosin. In addition, sections from some of the stomach implants were treated with McManus's periodic acid-Schiff stain while others were postchromed and then stained for pepsinogen granules using a modification of Bowie's technique (*5*). Tumors were diagnosed following microscopic examination.[4]

Experimental Procedures and Results

The mice were divided into three equal groups. Those in group A received implants of skin; in group B, the implants consisted predominantly of muscle tissue; and in group C, they were derived from the glandular portion of the stomach.

The following results were obtained with fetal skin after an average observation period of 199 days (group A, table 1). A tumor incidence of 80 percent was observed in the spleen, compared with 8 percent in the liver. The intrasplenic tumors were diagnosed as squamous-cell carcinomas (fig. 1) and as fibrosarcomas. The 3 intrahepatic tumors were all squamous-cell carcinomas. At autopsy, implants were recovered from the liver and spleen of many non-tumor-bearing mice in group A. All those from the spleen appeared viable microscopically, whereas only 7

[4] The author is grateful to Drs. Thelma B. Dunn and Leslie R. C. Agnew for their assistance.

TABLE 1.—*Tumors induced and fates of grafts of fetal tissues implanted, with methylcholanthrene, into strain C3H male mice*

Group	Number of mice*	Tissue transplanted	Average observation period (*days*)	Site of transplantation	Tumor incidence (*percent*)	Fibrosarcomas† Number (average size in cm.‡)	Squamous-cell carcinomas† Number (average size in cm.‡)	Nontumorous implants recovered: viable	Nontumorous implants recovered: nonviable	Implants not recovered
A	39	skin	199	liver	8		3 (1.1 × 0.9)	7	11	18
				spleen	80	3 (2.2 × 2.1)	28 (1.5 × 1.1)	7	0	1
B	40	muscle, etc.§	237	liver	5	1 (0.8 × 0.7)	1 (1.2 × 0.8)	15	17	6
				spleen	80	27 (1.5 × 1.1)	5 (1.4 × 1.0)	5	1	2
C	34	stomach	290	liver	3		1 (0.4 × 0.3)	6	2	25
				spleen	15		5 (1.8 × 1.1)	25	2	2

*Survivors at time first tumor was palpated in the group.
†Includes only neoplasms observed at site of transplantation
‡An average of the two largest diameters.
§Implants composed predominantly of muscle tissue from body wall and limbs. In latter instance, cartilage and bone were also included.

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of 18 implants in the liver appeared viable. In 18 of the non-tumor-bearing mice there was no sign of an implant in the liver, while only 1 implant was not recovered from the spleen (table 1).

All 40 of the mice that received transplants of fetal muscle were alive at the time the first tumor was palpated (group B). Tumors were observed in the liver in 2 of the mice and in the spleen in 32 during an average observation period of 237 days (table 1). The liver tumors included 1 fibrosarcoma and 1 squamous-cell carcinoma, while in the spleen there were 27 fibrosarcomas (fig. 2) and 5 squamous-cell carcinomas. Microscopic examination of a 0.3 mm. nodule in the liver of one of the mice in group B disclosed an area strongly suggestive of squamous-cell carcinoma. Because the latter could not be given an unqualified diagnosis, however, it has been omitted from the table. In addition to tumor-bearing mice, implants were recovered from many of the non-tumor-bearers. Fifteen of 32 implants in the liver, and 5 of 6 in the spleen appeared viable microscopically. The viable implants consisted predominantly of cartilage, bone, and fibrous connective tissue with only occasional remnants of muscle tissue. No implants were found in 6 of the livers and in 2 of the spleens.

There were 34 survivors when the first tumor was palpated in group C (implanted with fetal stomach). Five of the mice bore intrasplenic tumors while 1 of the 5 also had a tumor in the liver (table 1). All 6 of the growths were diagnosed as squamous-cell carcinomas. The 15 percent tumor incidence obtained with fetal stomach transplants in the spleen was considerably less than that observed following intrasplenic transplantation of skin or muscle (table 1).

Implants that appeared viable microscopically were observed in the livers of 6 of the non-tumor-bearing mice, while in another 2 the liver implants appeared nonviable. Implants were not observed in the livers of the remaining 25 mice in group C. Many of the non-tumor-bearing mice in this group bore intrasplenic implants at the time of autopsy; viable grafts were recovered from 25 mice, nonviable ones from 2 mice. Glandular elements were present in almost all the viable implants. Some contained cysts lined in places with only a single layer of low columnar epithelium. A portion of the limiting ridge was observed in several of the implants. The glandular areas in the gastric implants appeared to be composed of mucous cells, an observation confirmed with McManus's periodic acid-Schiff stain. All of 10 specimens chosen at random from group C showed a strong positive reaction with this stain. Additional sections cut from the same 10 tissue blocks and stained for pepsinogen granules (Bowie's technique) were uniformly negative. Régaud's fluid is generally employed as the fixative in this technique. The decision to test for pepsinogen granules in the present experiment was made after the recovered implants had been fixed in Tellyesniczky's fluid. However, it was observed that the latter fixative did not prevent demonstration of pepsinogen granules if the tissues subsequently were postchromed.[5]

[5] Agnew and Klein, unpublished observations.

Granules were clearly visible in sections of untreated fetal mouse stomach when these were fixed in Tellyesniczky's fluid.

Discussion

The resistance of both liver and spleen to the induction of fibrosarcomas with the carcinogenic hydrocarbons has been demonstrated repeatedly (*1*). Although it is possible some of the fibrosarcomas in groups A and B may have developed from host connective tissue, it is more likely that these neoplasms arose from the tissues transplanted; in view of the cell type concerned, the squamous-cell carcinomas observed in the liver and spleen undoubtedly arose from implanted and not from host tissues.

When fetal tissues were transplanted to the liver and spleen of each mouse, tumors arose in both sites in 6 of the recipients. Although it is possible that some of these tumors were metastases, this is unlikely for the following reasons: 1) Tumors induced with grafts of fetal mouse tissues in other experiments have rarely been observed to metastasize (*2*, *4*, *6*). 2) In the present experiment, when a mouse bore a tumor in both liver and spleen, the two neoplasms frequently were of different types. 3) When tumors of the same type were induced in both sites within the same host, tissue remains from the original implant generally were observed within or adjacent to each of the tumors.

The incidence of tumors induced with grafts of fetal tissues was five to sixteen times higher when transplantation was to the spleen as opposed to the liver (table 1). It is apparent from these data that the spleen is a more favorable site than the liver for the induction of tumors in fetal transplants. The low survival rate of implants in the liver undoubtedly contributed to the low tumor incidence observed in that organ. (The number of implants not recoverable at autopsy was considerably higher for the liver than for the spleen, as shown in table 1.) It is probable that more tumors would have been induced had more implants survived transplantation to the liver. The low incidence of intrahepatic tumors may also have been associated with a more rapid removal or elimination of carcinogen. It is well known that the rate of blood flow through the spleen is less than that through the liver. This would favor a more prolonged exposure of tissue to carcinogen in the spleen and thereby result in the induction of more tumors. The metabolism of foreign chemical compounds occurs to a large extent in the liver. If before exerting a tumorigenic effect some or all of the carcinogen was converted into a noncarcinogenic metabolite(s), this too would lower the incidence of intrahepatic tumors.

The poor survival of implants in the liver was unexpected in view of the rich blood supply and abundance of nutrients available. However, it is possible that vascularization was impeded because of the presence in the liver of metabolites and waste products injurious to the implants. Although an appreciable number of viable intrahepatic implants was recovered in group B (table 1), these were largely composed of cartilage, bone, and fibrous connective tissue. The ability of such tissues to with-

stand unfavorable conditions following transplantation probably accounts for their survival in the liver.

The resistance of the spleen to induction of fibrosarcomas appeared to have little effect on the development of these tumors in grafts of muscle tissue transplanted intrasplenically. However, in the liver, which also is refractory to fibrosarcoma induction, the same susceptible grafts produced only 1 such tumor (group B, table 1). Although the low tumor incidence in the liver may be explained on the basis of poor survival of the implants, the present data are insufficient to attribute the result to this effect alone.

Occasionally in preparing transplants for group B the skin parted, leaving fragments that adhered to the underlying tissues. It is possible that the squamous-cell carcinomas in group B were derived from such remnants that escaped detection and were incorporated in the grafts of muscle tissue.

A tumor incidence of 15 percent was observed following intrasplenic transplantation of fetal stomach and methylcholanthrene (MCA) (table 1, group C). Under similar conditions, however, an incidence of 80 percent was observed with grafts of fetal skin or muscle (groups A and B). These results, in themselves, do not necessarily indicate a greater tissue susceptibility for fetal skin or muscle, since a protective influence against MCA may have been exerted by the numerous mucus-secreting cells lining the encysted grafts of fetal stomach. Appreciable amounts of mucus were demonstrated within the cysts, using McManus's periodic acid-Schiff stain.

Six squamous-cell carcinomas were observed in the implanted stomach tissue in 5 of the mice in group C. Five of the neoplasms appeared in the spleen and 1 in the liver. Smith (*6*) injected MCA and the glandular portion of fetal mouse stomachs intramuscularly in mice and also obtained squamous-cell carcinomas. It appeared that these tumors originated as a result of metaplasia of columnar to squamous epithelium (*6*). A similar metaplasia has been observed in the mouse (*7*) and rat (*8*) following intramural injection of MCA in the glandular stomach. In the present experiment, several of the grafts recovered at autopsy contained a remnant of the limiting ridge. Under these circumstances, one or more of the squamous-cell carcinomas in group C may have arisen directly from forestomach epithelium rather than from metaplastic glandular epithelium. Some of the nontumorous implants contained nests of squamous cells surrounded by glandular epithelium. Here, too, it sometimes could not be determined whether this was the result of metaplasia or whether the squamous tissue represented an extension of forestomach epithelium. When the limiting ridge appeared in the same section, the latter appeared more likely.

Induction in the gastric glandular mucosa of squamous-cell carcinomas instead of adenocarcinomas has been attributed (*6*) to a rapid metaplasia of columnar to squamous epithelium. It has also been suggested that if the same tissue were exposed to a lower dose of MCA, this might retard

the onset of metaplasia and favor development of adenocarcinomas (*6*). In the present experiment, wherein a few crystals of MCA were employed with each graft, squamous-cell carcinomas but no adenocarcinomas were observed (table 1). However, since the carcinogen was applied in the form of crystals, some of the mucosal cells may have been subjected to an unusually high local concentration of MCA. Although metaplasia was observed in some of the grafts, many contained extensive areas of glandular tissue. Thus, it remains to be demonstrated whether or not exposure of gastric mucosal cells to a sufficiently low dose of MCA will retard metaplasia and encourage induction of adenocarcinomas. Since the period of observation ranged from about 200 to 400 days, it may be that this was not long enough for adenocarcinomas to develop within the transplants. On the other hand, gastric adenocarcinomas have been induced with MCA in strain C3H mice after much shorter intervals of time but following intramural injection (*7*). It is noteworthy that sarcomas were not observed when fetal stomach and MCA were transplanted to the liver or spleen, whereas tumors of this type were induced frequently in other experiments in gastric tumorigenesis (*6–8*).

In the experiments of Smith (*6*), parietal and chief cells in addition to mucous cells were observed after as long as 76 days in transplants of gastric epithelium exposed to MCA. Although tests for free HCl were negative, Smith (*6*) was able to demonstrate the presence of pepsin and rennin in the fluid contents of some of the cysts. Implants were recovered from the mice in group C after a much longer observation period (table 1). The glandular epithelium in these grafts was composed predominantly of mucous cells. Representative sections prepared from these implants and stained with McManus's periodic acid-Schiff stain all gave strong positive reactions typical of mucus-secreting tissues. However, sections from the same implants were uniformly negative when stained for pepsinogen granules. A similar persistence of mucous cells but a lack of chief cells was reported by Toovey *et al.* (*9*) and Kobernick *et al.* (*10*) following transplantation of a flap of gastric glandular mucosa to the anterior abdominal wall in the rat.

Summary

Grafts of fetal skin tamped with methylcholanthrene were homotransplanted to the liver and spleen of 40 C3H/Jax male mice. Two similar groups were treated in the same manner but with transplants of fetal muscle and glandular stomach, respectively. Among 113 mice that survived long enough for tumors to develop, 68 had intrasplenic tumors and 6 had intrahepatic tumors. The spleen, therefore, appeared to be a better site than the liver for induction of tumors with fetal transplants. A considerably higher rate of survival of the implants was observed in the spleen than in the liver. Among the mice implanted with fetal muscle, 27 bore intrasplenic fibrosarcomas, whereas but 1 tumor of this type was observed in the liver. The apparent resistance of the liver to induction of sarcomas as well as of carcinomas in susceptible transplants is discussed.

Although an effort was made to induce gastric adenocarcinomas by transplanting the glandular stomach, only squamous-cell carcinomas were obtained. Many of the non-tumor-bearing mice bore viable gastric implants, especially in the spleen. These implants contained extensive areas of glandular epithelium composed predominantly of mucus-secreting cells. Bowie's stain for pepsinogen granules gave uniformly negative results with the same implants.

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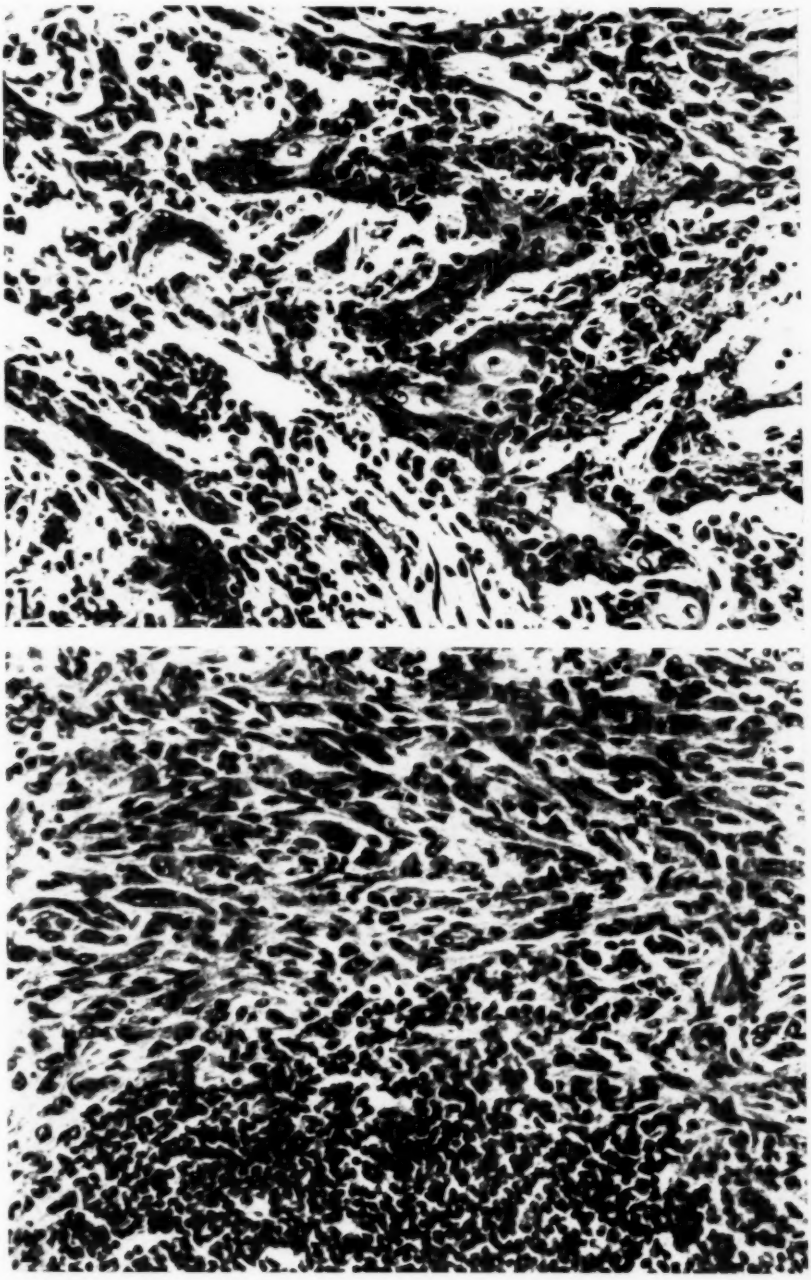
PLATE 85

FIGURE 1.—Squamous-cell carcinoma induced in the spleen following intrasplenic transplantation of fetal skin and methylcholanthrene. × 340

FIGURE 2.—Fibrosarcoma in the spleen observed following intrasplenic transplantation of fetal muscle and methylcholanthrene. × 340



Klein 1145

Further Observations on the Effects of Cancer Chemotherapeutic Agents on the *in Vitro* Dehydrogenase Activity of Cancer Tissue [1,2]

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The variations in the biologic behavior and response to therapy exhibited by different types of malignant tumors, as well as the differences among individual tumors of a similar type, are of major concern in therapy and prognosis. While the microscopic structure of a malignant neoplasm may sometimes be correlated with its clinical behavior, histologically identical tumors often differ in their response to treatment. Further, a tumor that was initially sensitive to a particular form of treatment may lose its sensitivity without appreciable change in microscopic structure.

In the hope that biochemical studies of tumor tissues might provide a more adequate method of evaluating such differences, we have been investigating the *in vitro* dehydrogenase activity of diverse cancer tissues (*1–3*). In a recent report we indicated some of the effects of urethan and triethylene melamine (TEM), singly and in combination, on the *in vitro* dehydrogenase activity of tumor tissues (*3*). It appeared from these data that there was a decided biochemical variability among cancer tissues from different patients having the same type of cancer. There was also a correlation between the clinically observed radiosensitivity of the tissue and the effect of urethan on the *in vitro* dehydrogenase activity of the tissue slice. The present paper is a report of our findings on an extended series of cases as well as a presentation of data on the effects of another cancer chemotherapeutic agent, aminopterin, employed singly and in combination with urethan and TEM.

Material and Methods

The tissue specimens were obtained from the operating rooms of the Flower and Fifth Avenue Hospitals within one-half hour of removal (in most cases within 10 minutes). The tissues were sliced freehand to a size of approximately 0.5 by 0.5 cm. and a thickness of about 1 mm., and the dehydrogenase activity was determined by means of the tetrazolium technique previously reported by us (*3, 4*). In table 1 we have listed the components and the concentrations of the various incubating solutions employed in this study. The intensity of the dehydrogenase activity in

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[2] This study was supported by the Damon Runyon Memorial Fund, The Leukemia Research Foundation, and the Helen Andreadis Foundation.

[3] With the technical assistance of Phyllis Brenowitz, B. A.

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TABLE 1.—*Composition of various incubating mediums. Components are indicated by concentration and volumes in milliliters*

Determination	1 Percent TTC* (*ml.*)	0.9 Percent saline (*ml.*)	0.9 Percent urethan (*ml.*)	0.5 Percent TEM† (*ml.*)	0.1 Percent aminopterin (*ml.*)
Endogenous (R_o)	3	1			
R_u	3		1		
R_{TEM}	3			1	
R_{TEM+u}	3		1	1	
R_{am}	3				1
R_{tr}	3		1	1	1

*2,3,5-Triphenyl tetrazolium chloride.

†The triethylene melamine (TEM) was generously supplied by Lederle Laboratories, Division of the American Cyanamid Company, through the courtesy of Dr. J. M. Ruegsegger.

the absence of any added inhibitor was taken as the endogenous dehydrogenase activity (R_o). The effects of the various chemotherapeutic agents on the *in vitro* dehydrogenase activity were obtained by determining the dehydrogenase activity in the presence of the test agent and then calculating the percent change from the R_o value.

As in our previous investigations, duplicate determinations were performed in almost all cases. In addition, examinations of frozen sections were made of representative samples of the tumor tissue that had been incubated in the various solutions employed. This provided an additional check on the quantitative determinations and also gave information regarding the degree of cellularity and necrosis. In confirmation of our previous observations there was no evidence that the percent inhibition of the dehydrogenase activity by the chemotherapeutic agents was related to the degree of cellularity, necrosis, or secondary inflammatory changes.

An attempt was also made to determine the degree of correlation between the effects of the various agents on the *in vitro* dehydrogenase activity, and their clinical therapeutic properties. A case was judged to be responsive to a therapeutic modality when treatment resulted in well defined regression of tumor masses and improvement in clinical status. If, on the other hand, similar treatment applied to cancer cases of the same type failed to alter the course of the disease and was without benefit, the case was then judged to be insensitive to that modality of treatment.

In those instances where radiation therapy was employed the treatment was exclusively determined by a qualified radiotherapist, in most instances by the Department of Radiology of the Flower and Fifth Avenue Hospitals. No case was considered insensitive to radiotherapy until the radiotherapist had indicated that his treatments were without benefit to the patient.

The chemotherapeutic studies were directly under the control of one of us (M. M. B.). All therapy and clinical control were personally directed and evaluated.

Results

Endogenous dehydrogenase activity.—In confirmation of our previously reported data a wide variation was found in the endogenous *in vitro* dehydrogenase activity of the various cancer-tissue slices. Such varia-

tion reflected differences in the intensity of the activity of the cancer tissue *per se* as well as the variable amounts of tumor tissue in the slices studied. We were unable to correlate the intensity of the endogenous dehydrogenase activity with the type of tumor, its microscopic appearance, clinical behavior, or response to therapy. Nor was there any difference noted when a comparison was made between the endogenous *in vitro* dehydrogenase activity of the primary tumor and of metastatic foci.

In view of the large numbers of cases in the present study, and since representative values for the endogenous dehydrogenase activity were indicated in our previous report, we shall omit a listing of the individual values in the present paper.

Urethan inhibition.—Table 2 indicates the distribution of 149 cancer cases according to the varying degrees of urethan inhibition of the *in vitro* dehydrogenase activity of the different types of cancer tissue. It is evident from these data that there is a decided heterogeneity in the degree of urethan inhibition indicating that, by this criterion at least, we are not measuring a factor of tissue metabolism common to all tumors.

TABLE 2.—*Distribution of varying degrees of urethan inhibition of* in vitro *dehydrogenase activity*

Diagnosis	Number of cases	Percent effect $\left(\frac{R_o - R_u}{R_o} \times 100\right)$			
		<−15 Percent	−15 to −29 Percent	−30 to −44 Percent	>−44 Percent
Adrenal carcinoma	2	1	1	.....	.....
Bladder carcinoma	4	2	1	1	.....
Brain tumor					
Meningioma	1	.....	.....	1	.....
Astrocytoma	1	1	.....	.....	.....
Breast carcinoma	62	28	13	12	9
Bronchogenic carcinoma	3	3	.....	.....	.....
Colon carcinoma	6	2	3	1	.....
Endometrial carcinoma	2	.....	1	.....	1
Epidermoid carcinoma					
Oropharynx	7	1	3	2	1
Gastric carcinoma	11	4	1	4	2
Hodgkin's disease	11	6	2	2	1
Leukemia, chronic					
Lymphatic	1	.....	1	.....	.....
Myelogenous	1	.....	1	.....	.....
Lymphosarcoma	8	2	2	2	2
Melanoma	5	4	1	.....	.....
Metastatic carcinoma, primary site undetermined	4	3	.....	.....	1
Neuroblastoma	2	.....	1	1	.....
Ovarian carcinoma	7	2	3	2	.....
Renal carcinoma	1	1	.....	.....	.....
Sarcoma					
Fibro-	4	2	2	.....	.....
Leiomyo-	1	1	.....	.....	.....
Osteogenic	1	.....	1	.....	.....
Testicle, embryonal					
Adenocarcinoma	3	1	2	.....	.....
Thymoma	1	.....	.....	1	.....
Total	149	64	39	29	17
Percent incidence	.....	43	26	19	11

That such heterogeneity may also occur among individual cases of the same cancer type is evidenced by 62 cases of breast carcinoma. In this latter series we also found a lack of homogeneity. In fact, the percentage incidence of the various degrees of inhibition are almost identical to that of the total series. These data are further indication that carcinoma of the breast may not only present differences in microscopic structure but also biochemical variations.

On the other hand, it is not to be inferred that a tendency toward a more homogeneous metabolic behavior does not occur in some tumor types. Thus, in 13 previously untreated and radiosensitive lymphomas (Hodgkin's disease and lymphosarcoma) the minimal inhibition by urethan was 14 percent, 6 of the cases were inhibited 30 percent or more and the mean percent inhibition was 36 percent. In contrast, of 5 melanoma cases tested none showed an *in vitro* inhibition as great as 30 percent. These data, as well as additional observations, will be discussed below in regard to the relationship between the radiosensitivity of cancer tissues and the urethan inhibition of their *in vitro* dehydrogenase activities.

Triethylene melamine (TEM).—Table 3 presents the distribution of the

TABLE 3.—*Distribution of varying degrees of TEM inhibition of* in vitro *dehydrogenase activity*

Diagnosis	Number of cases	Percent effect $\left(\frac{R_o - R_{TEM}}{R_o} \times 100\right)$			
		<−15 Percent	−15 to −29 Percent	−30 to −44 Percent	>−44 Percent
Adrenal carcinoma	1	.....	1	.....	.....
Bladder carcinoma	3	2	1	.....	.....
Brain tumor					
Meningioma	1	.....	.....	1	.....
Astrocytoma	1	1	.....	.....	.....
Breast carcinoma	48	22	13	8	5
Bronchogenic carcinoma	3	3	.....	.....	.....
Colon carcinoma	5	2	2	1	.....
Endometrial carcinoma	2	.....	1	1	.....
Epidermoid carcinoma					
Oropharynx	7	1	2	4	.....
Gastric carcinoma	7	4	1	1	1
Hodgkin's disease	8	3	2	2	1
Leukemia, chronic					
Myelogenous	1	.....	.....	1	.....
Lymphosarcoma	5	3	.....	2	.....
Melanoma	2	2	.....	.....	.....
Metastatic carcinoma, primary site					
undetermined	3	1	.....	2	.....
Neuroblastoma	2	1	.....	1	.....
Ovarian carcinoma	4	1	1	2	.....
Renal carcinoma	1	.....	.....	1	.....
Sarcoma					
Fibro-	3	2	.....	1	.....
Leiomyo-	1	.....	1	.....	.....
Osteogenic	1	1	.....	.....	.....
Testicle, embryonal					
Adenocarcinoma	2	1	.....	1	.....
Thymoma	1	.....	.....	.....	1
Total	112	50	25	29	8
Percent incidence	.....	45	22	26	7

degrees of TEM inhibition of the *in vitro* dehydrogenase activity of 112 cases of various types of cancer tissues. In this series TEM produced an inhibition of 30 percent or greater in 37 (33%) of the cases. On the other hand in 50 (45%) of the cases no significant inhibition was observed. It will be noted that the distribution of the *in vitro* effects of TEM was similar to that of urethan. However, it should also be mentioned that when a comparison was made of the degree of *in vitro* inhibition by urethan and by TEM, the following relationship was found. In approximately half of the cases the degree of inhibition by these two agents was similar, in one-fourth of the cases the urethan inhibition was greater than that with TEM, while the reverse was true in the remaining tissues.

Attention should also be called to the findings in the series of 48 cases of breast carcinoma. As with urethan, the TEM exhibited a marked variation in its effect on the *in vitro* dehydrogenase activity of the breast cancer tissue—further evidence of differences in metabolism among tumors of the same site of origin.

TEM plus urethan.—In table 4 we have indicated the distribution of the degrees of inhibition produced by the combination of urethan plus

TABLE 4.—*Distribution of varying degrees of inhibition of* in vitro *dehydrogenase activity by urethan plus TEM*

Diagnosis	Number of cases	Percent effect $\left(\frac{R_o - R_{u+TEM}}{R_o} \times 100\right)$			
		<−15 Percent	−15 to −29 Percent	−30 to −44 Percent	>−44 Percent
Adrenal carcinoma	1	1			
Bladder carcinoma	4	3	1		
Brain tumor					
Meningioma	1		1		
Astrocytoma	1	1			
Breast carcinoma	45	12	12	13	8
Bronchogenic carcinoma	3	1	1	1	
Colon carcinoma	5	3	2		
Endometrial carcinoma	2			1	1
Epidermoid carcinoma					
Oropharynx	7	1	1	4	1
Gastric carcinoma	8		1	5	2
Hodgkin's disease	8	2	1	4	1
Leukemia, chronic					
Myelogenous	1			1	
Lymphosarcoma	5	1	2	2	
Melanoma	2	1	1		
Metastatic carcinoma, primary site					
undetermined	2		1	1	
Neuroblastoma	2			2	
Ovarian carcinoma	4		1	1	2
Renal carcinoma	1			1	
Sarcoma					
Fibro-	3		1	1	1
Leiomyo-	1		1		
Osteogenic	1		1		
Testicle, embryonal					
Adenocarcinoma	2		1	1	
Thymoma	1				1
Total	110	26	29	38	17
Percent incidence		24	26	35	15

TEM on 110 cases of various types of cancer tissue. It will be noted that an inhibition of 30 percent or greater was found in 50 percent of the cases. Thus, the combination of urethan plus TEM produced an appreciable degree of inhibition more frequently than either urethan (30%) or TEM (33%) used singly.

In comparing the degree of inhibition produced in individual cases by the combination of urethan plus TEM with the inhibition produced by TEM and urethan singly, it was not possible to predict the combined values on the basis of the individual effects. In some cases appreciable inhibition was produced by the combination although no inhibition was found with either TEM or urethan used alone. In almost one-fourth of the cases tested, urethan plus TEM produced an inhibition at least 15 percent greater than was observed with either of the agents alone. On the other hand, in approximately 10 percent of the cases the degree of inhibition was at least 15 percent less when the combination was used as compared with that caused by one of the agents used alone. It thus appears that while this combination of agents tends to inhibit the *in vitro* dehydrogenase activity of cancer tissues more frequently and to a greater degree than does either agent used alone, such action is not universal and

TABLE 5.—*Distribution of varying degrees of aminopterin inhibition of* in vitro *dehydrogenase activity*

Diagnosis	Number of cases	Percent effect $\left(\frac{R_o - R_{am}}{R_o} \times 100\right)$			
		<−15 Percent	−15 to −29 Percent	−30 to −44 Percent	>−44 Percent
Adrenal carcinoma	2	2			
Bladder carcinoma	1	1			
Brain tumor					
Meningioma	1	1			
Astrocytoma	1	1			
Breast carcinoma	32	23	4	2	3
Colon carcinoma	4	4			
Epidermoid carcinoma					
Oropharynx	6	5			1
Gastric carcinoma	5	2	1	2	
Hodgkin's disease	6		3	3	
Leukemia, chronic					
Myelogenous	1	1			
Lymphosarcoma	3	3			
Melanoma	2	2			
Neuroblastoma	2			2	
Ovarian carcinoma	4		3	1	
Sarcoma					
Fibro-	2		1	1	
Leiomyo-	1	1			
Osteogenic	1	1			
Testicle, embryonal					
Adenocarcinoma	1	1			
Thymoma	1				1
Total	76	48	12	11	5
Percent incidence		63	16	14	7

is unpredictable in the individual case. This may be further evidence of the complexity and diversity of the metabolic activity of cancer tissue.

Aminopterin.—The effect of aminopterin on the *in vitro* dehydrogenase activity of 76 cases is indicated in table 5. It is readily apparent that aminopterin only infrequently caused appreciable inhibition in the cases studied. In 79 percent of the cases the inhibition was less than 30 percent.

Combination of urethan, TEM, and aminopterin.—It is evident from the data of table 6 that this triple combination produced effects similar to those of the combination of urethan and TEM. However, in 11 percent of the 75 cases tested the inhibition produced by the triple combination was greater than 30 percent, although the effects of the agents tested separately failed to inhibit the tissues appreciably. It would thus appear that in some instances this combination may produce *in vitro* effects that would not have been expected on the basis of the effects of the agents used singly. Conversely, in some cases it was found that the combination of the three agents failed to produce any appreciable inhibition of the *in vitro* dehydrogenase activity of the tumor slices despite the fact that one or more of the agents used alone had caused decided inhibition. This again provides evidence of the complexity and variation of the metabolism among individual cancer cases.

Relationship between in vitro *and clinical effects.*—In a previous report we indicated that the degree of *in vitro* inhibition by urethan was more closely correlated with the radiosensitivity of the tissue than with the microscopic

TABLE 6.—*Distribution of varying degrees of inhibition of* in vitro *dehydrogenase activity by combination of urethan, TEM, and aminopterin*

Diagnosis	Number of cases	Percent effect $\left(\frac{R_o - R_{tr}}{R_o} \times 100\right)$			
		<−15 Percent	−15 to −29 Percent	−30 to −44 Percent	>−44 Percent
Bladder carcinoma	1	.....	1	.....	.....
Brain, meningioma	1	.....	1	.....	.....
Breast carcinoma	35	3	8	15	9
Colon carcinoma	4	3	1	.....	.....
Epidermoid carcinoma, oropharynx	6	1	1	3	1
Gastric carcinoma	6	.....	2	.....	4
Hodgkin's disease	4	2	2	.....	.....
Leukemia, chronic myelogenous	1	1	.....	.....	.....
Lymphosarcoma	3	.....	1	2	.....
Melanoma	2	1	1	.....	.....
Neuroblastoma	2	2	.....	.....	.....
Ovarian carcinoma	4	1	.....	2	1
Sarcoma					
Fibro-	2	.....	1	.....	1
Leiomyo-	1	.....	.....	1	.....
Osteogenic	1	.....	1	.....	.....
Testicle, embryonal, adenocarcinoma	1	.....	.....	1	.....
Thymoma	1	.....	.....	.....	1
Total	75	14	20	24	17
Percent incidence	.....	19	27	32	23

TABLE 7.—*Percent of inhibition of* in vitro *dehydrogenase activity in relation to clinical response*

Diagnosis	X ray (urethan effect)		TEM		TEM+u	
	Sensitive† (percent)‡	Insensitive (percent)	Sensitive (percent)	Insensitive (percent)	Sensitive (percent)	Insensitive (percent)
Breast carcinoma	−55	+9	−50	.....	−51	.....
	−46	+3	.....	.....	−49	.....
	.....	−8	.....	.....	−41	.....
	.....	−20	.....	.....	.....	.....
	.....	−11	.....	.....	.....	.....
	−31	.....	.....	.....	.....	.....
Bronchogenic carcinoma	.....	.....	.....	.....	−36	−29
Colon carcinoma	.....	.....	.....	.....	.....	−19
Epidermoid carcinoma	.....	+30	.....	.....	−40	.....
	.....	−15	.....	.....	.....	.....
Fibrosarcoma	.....	+13	.....	.....	.....	.....
Hodgkin's disease	−61*	−11	−45	+54	−37	−8
	−43*	+36	−37*	+31	−43	−16
	−22*	+8	−41*	−16	−58*	.....
	−30*	−13	−27*	.....	−40*	....
	−14*	.....	−11*	.....	−34*	.....
	−21*	.....	.....	.....	−42*	.....
	−18*	.....	.....	.....	.....	.....
Kidney carcinoma	.....	−11	.....	.....	.....	−32
Leukemia, chronic myelogenous	−20*	−23	.....	−32	.....	.....
Lymphosarcoma	−46	+4	−41*	−11	−30	.....
	−71*	−8	+10*	−9	−38*	.....
	−37*	.....	−34*	.....	−20*	.....
	−30*	.....	.....	.....	−25*	.....
	−27*	.....	.....	.....	.....	.....
	−17*	.....	.....	.....	.....	.....
Melanoma	.....	.....	.....	.....	.....	−14
Neuroblastoma	−35	.....	−39	.....	.....	.....
Ovarian carcinoma	.....	−23	.....	.....	−38	.....
Mean	−34.7	−2.4	−31.5	+2.8	−38.9	−19.7

*Untreated lymphoma, presumed clinically responsive to therapeutic modality under consideration.
† Clinical response.
‡ Inhibition.

appearance of the tumor or its site of origin (*3*). Of the tissues tested thus far we have been able to compare the *in vitro* effects of various agents with the clinical response in 73 instances. The pertinent data in the individual studies are indicated in table 7. A comparison of the distribution of the degrees of inhibition in the clinically sensitive and insensitive groups is listed in table 8. Evaluation of the significance of these data is best made in terms of the individual groups.

Urethan.—In 17 cases the clinical use of X-ray therapy had failed to

TABLE 8.—*Distribution of varying degrees of inhibition of* in vitro *dehydrogenase activity in relation to clinical behavior*

Clinically Sensitive

Percent inhibition	X ray	TEM	TEM+u	Total
<−15 percent	1	2	0	3 (7%)
−15 to −29 percent	6	1	2	9 (20%)
−30 to −44 percent	6	5	11	22 (50%)
>−45 percent	5	2	3	10 (23%)
Total	18	10	16	44

Clinically Insensitive

Percent inhibition	X ray	TEM	TEM+u	Total
<−15 percent	13	4	2	19 (66%)
−15 to −29 percent	4	1	3	8 (28%)
−30 to −44 percent	0	1	1	2 (7%)
>−45 percent	0	0	0	0 (0%)
Total	17	6	6	29

produce any apparent benefit. Of these cases 6 were lymphomas (lymphosarcoma and Hodgkin's disease) which were no longer responsive to radiation therapy. It will be noted from table 7 that the urethan effect on the *in vitro* dehydrogenase activity of these cases varied from +36 percent to −23 percent with a mean of −2.4 percent. The distribution of values as indicated in table 8 differs markedly from that of the total series. As judged by the Chi-square method of testing, the probability (*P*) that such a difference would occur by chance is equal to 0.02.

In contrast, in those cases known to have had a good clinical response to radiation therapy, and in the untreated lymphomas (presumed radiosensitive), the *in vitro* inhibition by urethan varied between −14 percent and −71 percent with a mean value of −34.7 percent. A statistical comparison (Chi-square method) of the distribution of values for the urethan inhibition as found in the radiosensitive and radioresistant groups indicated that such a difference would occur by chance less than once in 1,000 times. These data give strong indication that the degree of urethan inhibition of the *in vitro* dehydrogenase activity is more closely correlated with the degree of clinical radiosensitivity than with the site of origin or the microscopic structure of the tumor. One might also note that of the 5 melanomas studied, a malignant neoplasm notorious for its radioresistance, the urethan inhibition of the *in vitro* dehydrogenase activity was uniformly low.

TEM.—In the present study clinical trials with nitrogen mustard or TEM were without benefit in 5 cases. In these cases the *in vitro* effect of TEM varied from +54 percent to −32 percent. It should also be noted that all of these cases were known to be radioresistant. In an additional radioresistant case of Hodgkin's disease, wherein data on the clinical effects of TEM was not available, TEM produced a +31 percent

change in the *in vitro* dehydrogenase activity. In contrast, in 3 cases wherein the mustards produced dramatic clinical benefit, TEM inhibited the *in vitro* dehydrogenase activity 39, 45, and 50 percent. In addition, in 5 of 7 cases of previously untreated lymphomas the TEM inhibition was greater than 25 percent. The mean value for the TEM inhibition in all 7 of the cases was 26 percent. Although the present series is small it is clear that the mustard-sensitive and -insensitive groups deviate in their distribution of values from the total series and from each other. Statistical comparison by the Chi-square method indicated that the probability (P) of such differences occurring by chance is less than 0.05.

TEM plus urethan.—The clinical usage of the combination of urethan plus TEM or HN_2 for diverse types of malignant neoplasia will be reported elsewhere (*5*). Suffice it to say at this time that we have observed objective and subjective benefit concomitant with the use of such therapy in cases insensitive to either radiation or mustard therapy used alone. Our present observations that the combination of urethan plus TEM produced a greater degree and more frequent incidence of inhibition of the *in vitro* dehydrogenase activity of various types of tumor tissue would be in accord with our clinical experience thus far. In the present study urethan plus TEM was used without clinical benefit in 6 cases. The *in vitro* measurements made on tissue specimens from these cases showed the effects of urethan plus TEM ranging from −8 to −32 percent with a mean value of −19.7 percent. In 9 cases the clinical usage of urethan plus TEM was associated with dramatic clinical benefit. The mean *in vitro* inhibition in these latter cases was 41 percent. The various individual values found in these cases are indicated in table 7. In addition, it should be noted that of 7 previously untreated or known radiosensitive lymphomas, which might be assumed to be sensitive to the combination of urethan and mustard, the mean inhibition was 37 percent. As judged by the Chi-square method of testing, the probability that the differences observed between the sensitive and the insensitive series would occur by chance is less than 1 in 1,000. It would appear from these data that the *in vitro* measurements might serve to presage the effect of such therapy in a goodly percentage of cases.

Aminopterin.—As a cancer chemotherapeutic agent aminopterin has far less applicability than X ray or the mustards. Its primary effect appears to be on acute leukemia, although the literature does include reports of sporadic benefit in various other types of cancer (*6*). Our *in vitro* measurements would not be inconsistent with such clinical experience since only 15 of the 76 cases tested showed any appreciable inhibition by aminopterin. However, to date we have had only 1 opportunity to perform *in vitro* measurements on the effects of aminopterin in a case where the drug was used clinically. This was a case of chronic myelogenous leukemia which had become resistant to all other forms of therapy and also failed to respond to aminopterin. The *in vitro* measurements were performed on splenic tissue, splenectomy being done because of massive splenomegaly. The aminopterin inhibition of the *in vitro* dehy-

drogenase activity was found to be only 6 percent. One would hesitate to make a positive statement without further individual case studies. However, in terms of the known clinical usage of aminopterin and the *in vitro* measurements made thus far one might say that the present data are not inconsistent with the possibility that the clinical effects of aminopterin might be predicted by *in vitro* measurements of the type described.

Discussion

The data obtained in the present investigation clearly indicate that, in terms of the types of measurements made, the metabolism of tumor tissue may exhibit marked variation from case to case. Such variations may also occur among individual cases of the same type of cancer. However, it is of interest to note that the degree of variation was much less when one grouped those cases known to have a similar clinical response to X ray or chemotherapy, regardless of the cancer type or individual microscopic structure. Thus, of 17 cases comprising 8 different types of malignant tumors, all known to be clinically radioresistant, all were biochemically similar in regard to exhibiting only minimal urethan inhibition of their *in vitro* dehydrogenase activity. A similar absence of any appreciable *in vitro* inhibition by urethan was also found in all of the 5 cases of melanoma. This latter observation is pertinent to the above discussion in view of the marked radioresistance of this type of cancer. Conversely, of 18 cases including 5 different tumor types, all of which showed, or would have been expected to show, a decided clinical radiosensitivity, the majority showed an *in vitro* urethan inhibition of 30 percent or greater. Statistical comparison of the distribution of degrees of urethan inhibition between the total series and the radiosensitive and radioresistant groups and between the latter two groups indicates that the differences are highly significant. It thus appears fair to state that: *cancer cases showing minimal urethan inhibition of the* in vitro *dehydrogenase activity would be less likely to experience benefit from X-ray therapy than those cases showing a high degree of* in vitro *inhibition by urethan.* However, it must be emphasized that the foregoing generalization must be considered tentative and is based only on the cases tested. This reservation is necessary since we have found (table 2) that of 11 gastric carcinomas tested 6 showed a urethan inhibition of 30 percent or more. It is generally held that these lesions are radioresistant. The exact significance of these data must await further study.

In regard to the findings with TEM, urethan plus TEM, and aminopterin, the data are at least consistent with the possibility that such *in vitro* measurements might presage clinical chemotherapeutic benefits. Certainly additional studies along these lines are indicated. The possibility that such studies might also be used to suggest compounds of potential cancer chemotherapeutic value appears worthy of investigation.

Summary

By means of the tetrazolium technique a study was made of the *in vitro* dehydrogenase activity of diverse types of human cancer tissues.

Measurements were made of the effect of urethan, TEM, and aminopterin, singly and in combination, on the *in vitro* dehydrogenase activity of the cancer tissues. An attempt was also made to correlate the *in vitro* effects of these agents with their clinical cancer chemotherapeutic effects. The data indicate that there is positive correlation between the *in vitro* effects of cancer chemotherapeutic agents tested and their clinical effects. It was also evident that there is marked variation in the metabolism of cancer tissues. The implications of these findings are discussed in relation to cancer therapy.

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The Growth of Pure Strain L Cells in Fluid-Suspension Cultures[1, 2]

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In previous papers, methods have been described by which it has been possible to discard the use of a plasma clot and to obtain massive growth of various fixed tissue cells on surface substrates such as cellophane and glass (*1–3*). Also it was possible to obtain luxuriant growth of cells on the surface of pyrex glass helices, massed together as a three-dimensional surface matrix, through the interstices of which the nutrient fluid could be circulated (*4*). When grown in this type of culture, relatively large amounts of living cells could be obtained; one culture contained over 1,100 mg. estimated wet weight of cells.

Earlier unpublished studies with the cells of the pure L strain had been made in this laboratory in an effort to obtain and clearly demonstrate cell proliferation in cultures of such design that the cells were freely suspended in a fluid culture medium without attachment to a surface substrate. These experiments were uniformly unsuccessful. In spite of these failures, frequent occurrence of dense cell suspensions in peritoneal ascites associated with carcinoma in the abdominal cavity suggested that cells might be made to live and proliferate in fluid suspensions *in vitro*. This was also suggested by the experimental transformation of lymphomas and carcinomas into ascites tumors *in vitro*. The recent successful growth by Owens, Gey, and Gey of cells of a lymphoblastoma in a "tumbling tube" culture *in vitro* (*5*) also suggested this possibility, although the use of lymphoblastoma cells did not exclude the possibility that cell proliferation in fluid *in vitro* might be a unique characteristic of cells of the lymphocytic series. Further, in both the tumbling tube culture and the ascites tumor *in vivo*, there was a possibility that actual cell proliferation occurred while the cells were adherent to some surfaces or some interfaces acting as surfaces. Thus, in the tube culture there appeared to be a possibility that during the actual proliferation phase the cells might adhere to the wall of the tube for a period, or even to a foam interface; with the ascites tumor,

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[3] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

too, there might be adhesion of the cell to some surface during actual mitosis.

In planning a restudy of this problem of growing tissue cells in fluid-suspension cultures, several factors were given special attention:

1) It was obviously necessary to keep the cells in suspension in the nutrient fluid. To assist in this an extremely slight increase in the viscosity of the nutrient fluid by some means such as addition of low concentrations of hyaluronic acid compounds or methylcellulose could be of substantial help. Purified hyaluronic acid compounds were not available in this study, so methylcellulose was used.

2) It was necessary to maintain adequate circulation of the culture fluid. With three-dimensional surface substrate cultures, earlier experiments had shown that an adequate rate of circulation of the nutrient culture fluid over the cells was of major significance for continued rapid cell proliferation (*4*). With a deep layer of a fluid suspension of cells, for maximum proliferation a rapid circulation of the fluid was certainly necessary in order to insure adequate oxygenation. Concurrently, rate and manner of circulation must be such as not to injure the cells mechanically.

3) It was necessary to start the cultures with an adequate cell population. Previously reported work on the growth of isolated single cells (*6*) had indicated that even the best fluid culture media now available must be altered or "conditioned" by the cells before rapid cell proliferation occurred. Owing to the necessary rapid circulation of the fluid in the suspension culture, with constant washing of fluid over all surfaces of the cells, it was probable that the actual necessary stage of "conditioning" of the nutrient fluid was extraordinarily critical and might require for its attainment a very high initial cell-population density.

The present paper describes experiments in which, by control of these various factors, it has been possible to establish rapidly proliferating fluid-suspension cultures of pure strain L cells from the mouse.

General Materials and Methods

General materials and methods used, including the incubator, temperature control and recording equipment, special rotating-type culture tubes, and multiple-drum rotating tube equipment have been described previously (*7*). In the present study the velocities of the rotating drums were all increased so that the rates of rotation for the 8 drums were, respectively, 18.7, 37.5, 75, 150, 300, 600, 1,200, and 2,400 revolutions per hour. Velocities used in each experiment are indicated in tables 1 and 2, and in text-figures 1, 2, and 3.

Methylcellulose used was Dow "Methocel," pharmaceutical grade, viscosity 4,000 centipoises. The methocel was first soaked in distilled water, then autoclaved, cooled overnight, restored to volume with sterile distilled water, and finally diluted with an equal volume of double-concentration Earle's saline sterilized by filtration. The resulting diluted stock solution was then used in preparation of the final culture solutions. Final concentrations used are indicated in table 1.

General materials and methods for setting up replicate cultures have been described previously (*8*). In experiment 1538, all components of the culture fluid were mixed together and this mixture was used as a suspending agent for the cells. The suspension was shaken by hand and aliquots hand-pipetted into the culture flasks. In all other experiments, the cells were suspended in the embryo extract and this cell suspension was measured out into the culture tubes into which the methocel and serum had already been measured from a burette and had been coated over the whole surface of the culture tube.

General materials and methods for enumerating nuclei of the cultures have been described previously (*9*). For the methocel-containing cultures, it was necessary to fix the culture in the citrate fixing fluid, centrifuge out the cells at 1,100 r.p.m., rewash with citrate fixative, recentrifuge, and finally make up to volume and enumerate the nuclei. In the instance of experiment 1546, in which 0.6 percent methocel was used, each culture had to be diluted to 250 ml. with citrate fixative on the first wash in order to get the nuclei cleanly thrown down. This difficulty in separating the nuclei for enumeration led to reduction of methocel concentration in later experiments.

During any one experiment there was no renewal of the culture fluid. In the later experiments phenol red, to make a concentration of 0.002 percent, was included in the culture fluid. In the three final experiments, although the culture fluid was not renewed, the *p*H change in the cultures was followed by change in the color of the phenol red, and the carbon-dioxide-rich supernatant gas in the culture vessel was periodically flushed out with air; the cultures were then resealed and reincubated.

Cells used were from T-60 (*10*) flask cultures of the pure strain L mouse cells, which originated from a single isolated cell in culture (*6*). These strain L cells have now been maintained in a medium composed of dilute horse serum and chick-embryo extract for more than 12 years; they have been in a medium composed of dilute horse serum and hyaluronidase-treated, ultracentrifuged chick-embryo extract (*11*) for more than one year. This latter nutrient mixture was used throughout the present study.

Experiments and Results

Eight experiments were carried out. Data from the first five are presented in sections A, B, C, D, and E of table 1. These were exploratory experiments to obtain orienting concepts as to which factors were the more significant.

In experiment 1538 the single tube culture studied for 48 hours showed within that time a rise in the number of nuclei from the original level of 4,976,000, as determined on two aliquots of the original inoculum, to 9,761,000. There were numerous free-floating cell flocculi, but relatively few cells on the glass surface.

In experiment 1540, at 48 hours, on nuclear enumeration all cultures showed at least some increase in population. Cultures without methocel showed some granular, free-floating cell clumps, but most of the cells

TABLE 1.—*Data from five orienting experiments*

Section	Experiment No.	Nutrient fluid volume (*ml.*)	Speed of rotation of culture tube (*r.p.h.*)	Methocel concentration (*percent*)	Time (*hours*)	Nuclei count of individual cultures (*thousands*)			Average number of nuclei per culture (*thousands*)
A	1538				0	4748	5205	.....	4976
		20	37. 5	0. 2	48	9761	.....	.....	9761
B	1540				0	6278	6151	6674	6368
		10	37. 5	.....	48	11144	12754	11314	11738
		10	37. 5	0. 2	48	9253	12169	6942	9455
		20	37. 5	.....	48	8748	8818	7067	8211
		20	37. 5	. 2	48	6204	7636	.....	6920
(Remaining culture transferred to 1200 r.p.h. drum at 48 hours.)		10	1200. 0	.....	72	6578	.....	.....	6578
		10	1200. 0	. 2	72	6898	.....	.....	6898
		20	1200. 0	.....	72	4622	.....	.....	4622
		20	1200. 0	. 2	72	9440	.....	.....	9440
C	1546				0	5111	5400	5107	5206
		10	37. 5	.....	41	7662	8000	9631	8431
		10	37. 5	.....	72	3018	2982	1604	2535
		10	37. 5	0. 6	41	7138	8373	7493	7668
		10	37. 5	. 6	72	3609	3493	4564	3889
		10	300. 0	.....	41	9938	9982	8551	9490
		10	300. 0	.....	72	2169	3669	2711	2850
		10	300. 0	. 6	41	8800	8756	9004	8853
		10	300. 0	. 6	72	3249	2551	2791	2864
		10	1200. 0	.....	41	10720	9938	11484	10714
		10	1200. 0	.....	72	2867	1782	1580	2076
		10	1200. 0	. 6	41	8818	10364	10273	9819
		10	1200. 0	. 6	72	2858	2164	2728	2584
D	1558				0	4249	2791	4271	3770

		10	18.7	.....	25	2640	5247	3360	3748
		10	300.0	.....	25	4780	5900	5333	5338
(Phenol red used.)		10	2400.0	.....	25	5287	5500	5747	5511
		10	18.7	0.1	25	4597	4229	4757	4528
		10	300.0	.1	25	5933	6258	5533	5908
		10	1200.0	.1	25	8738	8293	6173	7735
		10	2400.0	.1	25	5713	3067	3298	4026
		10	18.7	.2	25	4053	2835	4405	3765
		10	300.0	.2	25	5213	5453	.....	5334
		10	2400.0	.2	25	.....	.....	.....	no cells
E	1577				0	1843	1927	1836	1868
		10	18.7	.....	22	1853	1799	1664	1772
		10	300.0	.....	22	1837	1310	1526	1557
		10	2400.0	.....	22	1693	1375	1652	1573
(Phenol red used.)		10	18.7	0.1	22	1581	1785	1432	1599
		10	300.0	.1	22	1787	1373	1581	1580
		10	2400.0	.1	22	1832	1490	1508	1610
					0	3686	3854	3672	3737
		10	18.7	.....	22	3185	3767	4080	3677
		10	300.0	.....	22	4036	4320	3760	4039
		10	2400.0	.....	22	4056	3855	4180	4030
		10	18.7	0.1	22	4065	3405	3350	3607
		10	300.0	.1	22	4511	3197	4657	4122
		10	2400.0	.1	22	4171	4076	4269	4172

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were attached to the glass as small masses or areas. In the methocel-containing cultures there were definitely more free-floating granular clumps of cells; in the cultures containing 10 ml. of nutrient fluid many cells and small masses still adhered to the glass; with the 20-ml. volume nearly all cells were free-floating in the fluid and the glass surface showed only a few attached cells. One culture under each set of experimental conditions was shaken slightly by hand and returned to the 1,200 r.p.h. drum. Twenty-four hours later these cultures all showed the cells aggregated in the form of small masses or granule-like particles floating freely in the fluid. Only a very few cells were attached to the glass, singly or in small granular masses. At this time the average nuclear population level had lowered. Data did not indicate whether the lowering was due to higher velocity of rotation or to exhaustion of the medium.

In experiment 1546, at 41 hours, cultures rotating at 37.5 r.p.h. with no methocel showed heavy sheets of cells adhering to the glass surface, but with many small, floating, granular masses of cells. At 300 r.p.h. there were many floating cells in small granular masses and cells on the glass were fewer and tended to be more in sheets. At 1,200 r.p.h. the sheets of attached cells were very thin; a larger number of the cells were floating freely in small granular masses. At 37.5 revolutions the methocel-containing cultures showed small areas of cells adhering to the glass, with many small granular masses of cells floating freely in the fluid. At 300 r.p.h., no cells could be seen adhering to the glass; all appeared as finely granular masses floating in the fluid. At 1,200 r.p.h. there were no adhering cells seen, and the free-floating clumps and masses were smaller, usually less than 0.2 mm. in diameter. At this time the nuclear population level in the 1,200 r.p.h., methocel-containing cultures averaged 9,819,000 as compared with an original inoculum level of 5,206,000. Cultures studied at 72 hours showed a greatly reduced nuclear population level, apparently due to necrosis resulting from exhaustion of the medium, since many necrotic and disintegrating nuclei were seen. The 300 and 1,200 r.p.h. cultures still showed no attachment of cells to the glass. The former showed the cells in the form of fine granules suspended in the fluid. These cell masses were so fine that when the tubes were upright there was no appreciable settling of the cells out of the fluid in 20 minutes. In the 1,200 r.p.h. cultures the cell granules or masses were smaller, ranging from single cells to granules about 0.1 to 0.2 mm. or less in diameter. When the tube was placed in an upright position there was no appreciable settling out in 25 minutes.

In experiment 1558 the duration of the study was reduced to 25 hours in the hope of being able to demonstrate a rapid cell proliferation uncomplicated by cell necrosis from exhaustion of the medium. At 18.7 r.p.h., with no methocel, the cells were largely adherent to the glass, in sheets and clumps of various sizes. At 300 r.p.h. the cell adhesion to the wall was less uniform and tended to occur in patches. More cells were in suspension in small masses and flocculi. At 2,400 r.p.h. there was a thin, more uniformly distributed sheet of cells on the glass; there was a heavy sus-

pension which showed a few small masses or flocculi. In the 18.7 r.p.h. group of cultures that contained either 0.1 or 0.2 percent of methocel, the cell distribution was indistinguishable from the 18.7 r.p.h. cultures without methocel. At 300 and 2,400 r.p.h. the appearance of the cultures was very similar to those at a corresponding velocity but without methocel, although with the methocel there were fewer cells attached to the wall. In cultures containing 0.2 percent of methocel, there was an even further reduction in the number of cells adhering to the wall; at 300 and 2,400 r.p.h. extremely few cells adhered to the wall; most of the cells were suspended as individual cells and small cell masses. At 25 hours the majority of these cultures showed some increase in nuclear population over the original population level. At 34 hours, in another group of cultures not detailed in the table but similarly treated, the cultures appeared to have passed their prime since they showed a sharply lower level in the several cultures counted; all cultures at this time showed a great deal of cellular disintegration. The nuclear enumeration on this group at 34 hours was therefore not completed. From 25 hours on, the *p*H in all cultures appeared to be below 6.7. In this last experiment the cultures had shown only a limited increase in the nuclear population level at 25 hours and a severe cellular degeneration at 34 hours. In the next experiment an attempt was made to lessen cell disintegration by shortening the time of the experiment still further.

In experiment 1577 two different inoculum population levels were used. The results were essentially similar. At 18.7 r.p.h., with no methocel, the cells were in thin patches on the glass and were floating in the fluid in loose flocculi up to $5 \times 5 \times 1$ mm. There were very few small suspended cell masses and extremely few loose suspended cells. At 300 r.p.h. there were some limited areas of thin cell sheets on the glass; there were no large, floating cell flocculi; the cells appeared to be largely aggregated in small free-floating granular or spherical masses of about 0.5 average and 1.0 mm. maximum size. At 2,400 r.p.h. there were no flocculi. There appeared to be no cells attached to the glass. There was a dense floating population of single cells and aggregates of several cells. Cultures that received methocel at 18.7 r.p.h. looked very much like those without methocel. There were a few small areas on the glass and some large floating cell flocculi, but no small cell aggregates floating in suspension. At 300 r.p.h. there were a great number of free-floating clumps of cells about 0.3 mm. in diameter; there was practically no adhesion of the cells or cell clumps to the glass. At 2,400 r.p.h. the cultures showed a dense suspension of free-floating isolated cells and small aggregates up to about ten cells. There was no increase in the nuclear population level at 22 hours as compared with that in the original inoculum. The *p*H of the cultures was approximately 6.9.

In the preceding experiments, results had indicated a high velocity of rotation as a major factor in maintaining a fine suspension of the cells in the culture fluid. The methocel applied to the glass appeared to have a definite role in keeping the cells from attaching to the glass. However,

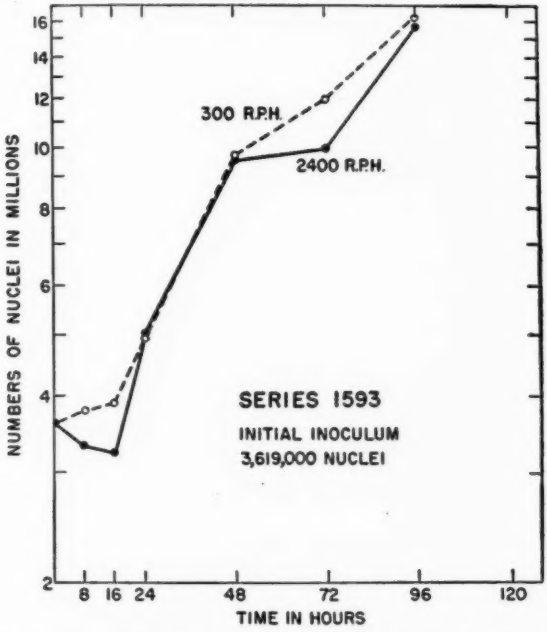
progressive shortening of the experimental interval, instead of giving more conclusive evidence of cell proliferation in suspension, had given less. It therefore became necessary to follow changes in culture populations over more extended periods of time.

Three final experiments, numbered 1593, 1608, and 1649 respectively, were therefore carried out. In cultures of each of these experiments the initial *p*H was 7.2 to 7.4. In these cultures nuclei were enumerated at

TABLE 2.—*Data from three final experiments*

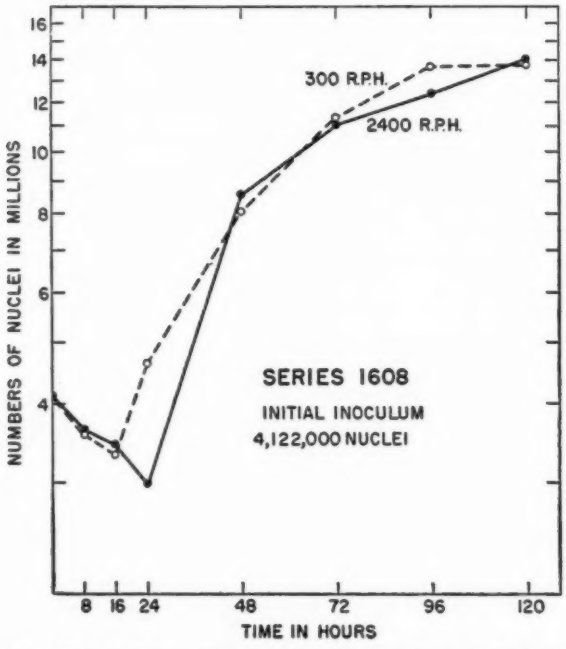
Experiment No.	Speed of rotation of culture tube (r.p.h.)	Time (hours)	Nuclei count of individual cultures (thousands)			Average number of nuclei per culture (thousands)
1593	.....	0	3617	3763	3478	3619
	300	8	4285	3866	3188	3780
	300	16	3687	4063	3870	3873
	300	24	4553	5156	5261	4990
	300	48	10052	10304	9014	9790
	300	72	12395	11984	11648	12009
	300	96	16171	16984	16104	16420
	2400	8	3209	3147	3585	3314
	2400	16	3253	2785	3670	3236
	2400	24	5196	5074	4853	5041
	2400	48	9380	9104	10584	9689
	2400	72	9618	9229	11059	9968
	2400	96	16075	17512	13448	15678
1608	.....	0	4307	3743	4316	4122
	300	8	3338	3844	3490	3557
	300	16	3407	3247	.....	3327
	300	24	4219	5062	.....	4641
	300	48	7140	8353	8696	8063
	300	72	12565	11040	10660	11422
	300	96	12736	14645	13227	13536
	300	120	12813	15053	13212	13693
	2400	8	3475	3880	3409	3588
	2400	16	3924	3379	3055	3453
	2400	24	2991	.....	.....	2991
	2400	48	8140	8320	9181	8547
	2400	72	10520	11872	10870	11087
	2400	96	12215	12864	12299	12459
	2400	120	13375	13763	15040	14059
1649	.....	0	3384	3028	2862	3091
	300	8	3388	3222	.....	3305
	300	16	2920	3443	4133	3499
	300	24	4690	3882	4349	4307
	300	48	7707	7280	8595	7861
	300	72	8536	9929	10283	9583
	300	96	6369	10987	9340	8899
	2400	8	2920	2993	2990	2968
	2400	16	2934	2449	2924	2769
	2400	24	4742	4064	4280	4362
	2400	48	7360	8604	6844	7602
	2400	72	7432	10200	9186	8939
	2400	96	10795	8574	9600	9656

various periods from 0 to 96 or 120 hours. In each experiment each culture received 10 ml. of fluid medium and to this was added 0.1 percent of methocel. In each series, groups of cultures were subjected to rotation speeds of 300 and 2,400 r.p.h. respectively. The original inoculum levels and changes in the nuclear population levels for individual cultures of each of these experiments are shown in table 2, and are summarized in text-figures 1, 2, and 3 respectively.



TEXT-FIGURE 1.—Changes in the numbers of nuclei in cultures of series 1593 plotted on semilogarithmic grids against time in hours. Each point represents the average of 3 cultures enumerated. The dotted line represents cultures at 300 r.p.h.; the solid line represents cultures at 2,400 r.p.h.

In each of these experiments, as determined by microscopic examination in all cultures rotating at 300 r.p.h., some cells continued to adhere to the glass tube; most of them were free-floating in the nutrient solution. While there were some free-floating, isolated, single cells, there were many small clumps or roughly spherical cell masses ranging from 0.1 to 0.5 mm. in diameter. When rotation was stopped and the flasks placed upright, rapid settling of these aggregates occurred. In cultures rotating at 2,400 r.p.h. no cells could be seen adhering to the glass wall of the tube; practically all of the cells were dispersed as single, isolated, rounded cells suspended in the nutrient fluid, or else as small clumps of from 2 to 10 cells suspended in the fluid. When rotation was stopped and the flasks were placed upright, settling of the cells was very slow. Final



TEXT-FIGURE 2.—Changes in the numbers of nuclei in cultures of series 1608 plotted on semilogarithmic grids against time in hours. Each point represents the average of 3 cultures enumerated, with the exceptions shown in table 2. The dotted line represents cultures at 300 r.p.h.; the solid line represents cultures at 2,400 r.p.h.

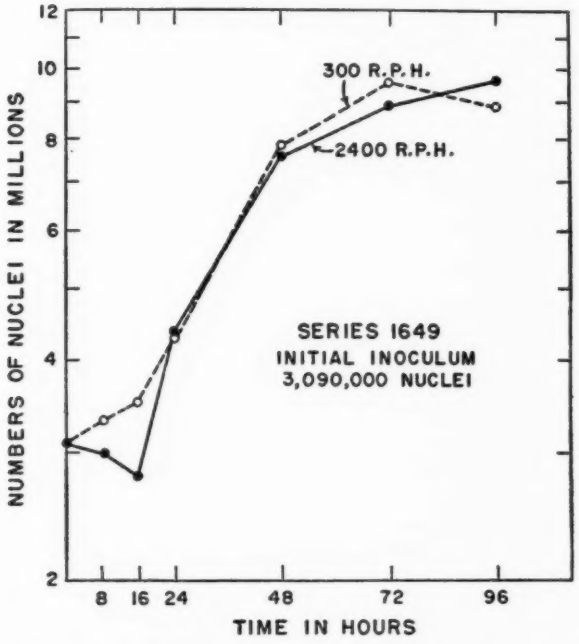
*p*H of all cultures was 6.8 to 7.0. No appreciable foaming was observed in any cultures.

Discussion

For this cell strain, at least, the results obtained indicate that fixed tissue cells can be grown in fluid suspension under conditions closely comparable to those now used for large-scale growth of bacteria and fungi. The nature of the variables controlled under the experimental conditions used are such as to justify the hope that comparable methods may also be used for other types of tissue cells.

Results obtained indicate that the methylcellulose, in the low concentration used, definitely reduces the adhesion of the cells to the walls of the glass culture tube, and also helps keep the cells in suspension. However, the high velocity of fluid circulation appears to be even more significant, both in keeping the cells off the glass and in keeping them in suspension. For practical large-scale cultures where it is no longer necessary to demonstrate the proliferation of cells without adhesion to the glass wall of the container, methylcellulose may possibly even be omitted entirely from the nutrient solution.

Within the range of roller-tube velocities studied, it appears that the size of the free-floating cell aggregates is directly correlated with the



TEXT-FIGURE 3.—Changes in the numbers of nuclei in cultures of series 1649 plotted on semilogarithmic grids against time in hours. Each point represents the average of 3 cultures enumerated, with the exception shown in table 2. The dotted line represents cultures at 300 r.p.h.; the solid line represents cultures at 2,400 r.p.h.

velocity of culture rotation; at 2,400 r.p.h. most of the cells existed as a suspension of single cells and aggregates of up to about 10 cells floating freely in the nutrient fluid; no large aggregate occurred at this velocity.

The rapid increase in the nuclear population level of the tube cultures rotating at 2,400 r.p.h., together with the absence of appreciable foaming or of cells adherent to the glass wall, shows that after the first 24 hours there is a rapid proliferation of cells floating freely in the nutrient fluid. No studies have yet been made on the mechanism by which the later stages of the separation of the daughter cells is accomplished. It is possible that the fluid agitation itself is a definite factor in accomplishing this final separation. It is also possible that in the small cell aggregates adhesion of a cell to other cells of the mass may have had some role.

Examination of text-figures 1, 2, and 3 shows that during the first 16 hours there was an increase in cell population in only two of the six groups of cultures presented in these figures; in the remaining four groups there was a sharp drop in population. The occurrence of this initial lag phase, or drop, is what could be expected experimentally if the original working concept of the cells having to alter or adapt the culture fluid to their needs were correct. This interpretation is also in agreement with the observation that in all three of these figures the cultures that were sub-

jected to a higher velocity of circulation, and consequently had the nutrient fluid washing more rapidly over the surface of the cells, consistently showed a sharper drop in population during the lag period than did cultures subjected to a slower rate of circulation.

This interpretation of the cause of the initial lag in cell proliferation is also in agreement with working concepts followed in obtaining proliferation from single isolated cells (*6*) by restricting diffusion around the cell by enclosing it in a capillary tube, and by using a preconditioned medium from a rapidly proliferating large culture on the cells. It is also in accord with earlier results which showed that a certain minimal number of cells per volume of nutrient is necessary for reliable growth of strain L cultures (*12*). In spite of these indications that some "conditioning" of the currently used medium by the cells is advantageous or necessary, no information has as yet been obtained as to the nature of this alteration or "conditioning."

The presence of this early lag or adaptation interval does clarify the confusing results from the preliminary experiments in which attempts were made to shorten the duration of time for each experiment. This same adaptation phenomenon also probably accounts for failures in earlier efforts to obtain fluid-suspension cultures. These results emphasize the apparent necessity of starting the fluid culture, in particular, with a large inoculum for each unit volume of nutrient fluid.

Summary and Conclusions

A study was made of the possibility of obtaining rapid proliferation of fixed tissue cells maintained as a suspension of cells floating in the nutrient culture fluid. Strain L cells were suspended in 40 percent horse serum, 20 percent 1:1 embryo extract from 10-day chick embryos, and 40 percent Earle's saline. A small amount of 4,000 centipoise methylcellulose was added to increase slightly the viscosity of the culture fluid and so facilitate keeping the cells in suspension. Cultures were maintained in special roller tubes rotating at various rates from 18.7 to 2,400 r.p.h. Changes in cell population were determined by nuclear enumeration methods previously described.

Early orienting experiments carried for 1 to 3 days usually showed substantial cell proliferation, but were not satisfactory in clearly defining the factors involved.

In a final group of 3 experiments in which the cultures were rotating at 300 and 2,400 r.p.h. respectively, the cultures were carried for longer intervals up to 5 days, while changes in the cell population level were periodically determined. These experiments were successful in demonstrating clearly proliferation of cells suspended in the culture fluid. Following a lag phase of up to 24 hours, there was a rapid proliferation of cells within the cultures. Microscopic examination showed that no cells were adhering to the surface of the glass tube.

From the results obtained the working concept is advanced that three factors require special attention in these fluid-suspension cultures. These factors are:

1) A slight increase of viscosity of the culture fluid. While this may not be essential, it is helpful in maintaining the cells in suspension.

2) A satisfactory type and amount of agitation or circulation of the cell suspension. This is necessary to maintain the cells in suspension and is probably necessary to insure adequate oxygenation and flow of nutrient over the cells.

3) It is probably necessary to have a starting cell population adequate to overcome the initial lag phase of the culture.

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Gonadotrophic Hormone Secretion from Hydatid Mole Grown in Tissue Culture [1,2]

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Little or no quantitative evidence has been available on the continued metabolic function of cells grown in tissue culture for extended periods of time. Since the production of hormones from tissue maintained in tissue culture can be used as an index of the specific metabolic activity of the cells, the successful cultivation of hydatid mole tissue over a prolonged period offers an excellent opportunity to follow such a metabolic activity as gonadotrophin production. Furthermore, the use of a sensitive bioassay method for gonadotrophins makes it possible to quantitate this hormone production. The purpose of this paper therefore is to report on the metabolic activity, as indicated by the amount of gonadotrophins produced, of tissue from several hydatid moles cultured for prolonged periods.

Material and Methods

Source of Material and Method of Planting

The hydatid mole tissue was removed surgically from patients at local hospitals.[6] The tissue was obtained from the uterus in three cases and from the peritoneal cavity in the fourth.[7] The material was brought to the Tissue Culture Laboratory within one hour of removal, and was then washed in culture fluid composed of 20 percent chick-embryo extract (1:1), 40 percent horse serum, and 40 percent Earle's saline (*1*), containing 400 units of penicillin and 400 units of streptomycin per ml. Each

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[2] This paper is based on a thesis prepared by the senior author in partial fulfillment of the requirement for a M.S. degree at George Washington University, May 1953. The paper was presented in preliminary form at the Annual Meeting of the American Association for Cancer Research, Chicago, Ill., April 1953.

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[5] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

[6] The authors wish to thank Dr. Marvin P. Footer, Dr. William B. Smith, and Dr. Morton S. Kaufman for obtaining the hydatid moles used in this study.

[7] The criteria for the establishment of the diagnosis in each case are those outlined by Crossen and Crossen (CROSSEN, H. S., and CROSSEN, R. J.: Diseases of Women. St. Louis, C. V. Mosby Co., 1935). The specimen recovered from the peritoneal cavity was an implant apparently left at the time of the initial hysterotomy.

vesicle used was cut into pieces about 3 by 12 mm., and each piece was placed in the culture fluid in the cavity of a depression spot plate. In all but the first experiment each piece was weighed on a torsion balance. Tissue pieces of comparable weights from the same bleb were paired; one piece was frozen for assay and the other was planted. Care was taken at all times to keep the tissue moist. Each strip of tissue to be cultured was placed under perforated cellophane (*2*, *3*), soaking in 0.5 ml. of plasma in a modified D-3.5 Carrel flask (*4*). The plasma was clotted with 0.75 ml. of the supernatant culture fluid. In preliminary experiments and in the second and third series, flasks containing the culture fluid only were maintained throughout.

Methods of Maintaining Cultures

Frequent observations were made to determine the amount of growth, the condition of the culture, and representative areas for photography (*5*, *6*). The nutrient fluids were renewed three times weekly. The used fluid was collected in a separate, sterile test tube and kept at −5° C. until assayed. At each fluid renewal, 0.5 ml. of fresh medium was added.

Transfer of Culture to Second Culture Generation

In the first experiment, two cultures were transplanted to the second generation in the following manner: The fluid was removed and retained in a pipette. The cellophane and explant were then transferred into a clean flask. The old plasma clot was scraped and removed from the original flask, which still contained a few cells adherent to the glass floor. The fluid in the pipette was divided between the two flasks and each was carried as a separate culture.

Method of Assay

A modified Levin-Tyndale test was used (*7*). Immature female Swiss mice weighing from 7 to 9 grams were used in the preliminary studies; later, mice weighing between 6 and 8 grams were found to be more sensitive assay animals. Three animals were used for each assay. Immediately before the injections, the culture fluid was thawed at room temperature and mixed thoroughly. All dilutions were made with triple-distilled water. One-half ml. of the fluid to be assayed was injected subcutaneously, daily for three days. In some cases undiluted fluid was injected and in three cases of low activity the daily dose was increased to one ml. Twenty-four hours after the last injection the mice were killed by cervical dislocation; the uterus was removed, freed of fluid, and weighed to the nearest milligram. An assay result was considered positive if two of the three test animals of a group had uterine weights twice the mean of the uterine weights of the untreated group. Fluid collected at intervals from control cultures, containing no cells but exposed to identical culture conditions, was also assayed.

The pieces of frozen tissue, paired with those grown in tissue culture, were assayed by the same method. Each was ground in a Potter-Elvehjem

homogenizer and serially diluted with distilled water. The smallest amount of mole-tissue homogenate (μg.) that would cause a positive uterine response was considered the threshold dose, or mouse uterus unit (M.U.U.), for that particular tissue pair.

The number of mouse uterus units in the fluid collected serially from each culture was calculated as follows: The volume of fluid for each collection period was multiplied by the highest dilution that would cause a positive response. This number was divided by 1.5 since three daily injections of 0.5 ml. each were used in the assay procedure. Stated in formula form:

$$\text{Mouse Uterus Units} = \frac{\text{No. of collections} \times \text{vol. per collection (ml.)} \times \text{dilution}}{\text{Total mouse dose (ml.)}}$$

$$\frac{4 \times 0.5 \times 300}{1.5} = 400 \text{ M.U.U.}$$

The percent increase in hormonal activity of the tissue of the culture is considered to be the increase in the number of mouse uterus units actually obtained from the fluid collected from the culture in excess of the number of mouse uterus units calculated on the basis of the initial weight of transplanted tissue. To eliminate the possibility that estrogenic substances might be stimulating the uterus directly, both the collected fluids and the homogenates of the mole tissue were frequently tested by injection into castrated female mice.

Experiments and Results

Gonadotrophic Activity

Series I.—Exploratory assays on fluid pooled from several cultures indicated the presence of large amounts of gonadotrophin.

Fluid from one culture contained gonadotrophic hormone after the tissue had been maintained *in vitro* for 413 days; fluids from two others were active at the end of 377 days.

Series II.—Table 1 summarizes the quantitative results of assays on cultures in Series II. The mole used in this series showed the least amount of gonadotrophic activity of the four studied. One culture was active for almost five months. No activity was found in another.

TABLE 1.—*Summary of gonadotrophin assay results for Series II*

Culture No.	Weight of tissue explant (*mg.*)	Threshold dose of homogenized tissue (*μg.*)	Calculated activity of explant (*M.U.U.*)	Total culture-fluid activity (*M.U.U.*)	Percent increase in activity
1	19. 4	198	98	169. 0	71
2	16. 2	198	82	338. 0	309
3	10. 1	198	51	305. 0	498
4	15. 6	150	104	267. 0	156
5	18. 7	150	125	4. 6	0
6	11. 1	150	74	0. 0	0

No estrogenic activity was obtained from doses of 300 μg of tissue homogenate.

Series III.—The quantitative results of this series are given in table 2. Five vesicles were bisected to give five paired pieces of tissue; the tissue homogenate had an average threshold activity of 63 μg. Three cultures from this mole were maintained for 135 days.

Doses of 750 μg. of the fresh tissue homogenate were assayed for estrogenic activity. All results were negative.

TABLE 2.—*Summary of gonadotrophin assay results for Series III*

Culture No.	Weight of tissue explant (*mg.*)	Threshold dose of homogenized tissue (*μg.*)	Calculated activity of explant (*M.U.U.*)	Total culture-fluid activity (*M.U.U.*)	Percent increase in activity
1	7.9	63	125	425	240
2	11.2	63	178	1,927	983
3	13.2	63	208	2,482	1,093
4	18.1	63	287	1,471	413
5	16.2	63	256	1,838	615

Series IV.—The material used in this series was obtained from the peritoneal cavity of the same patient from whom tissue was obtained six months earlier for Series III.

Threshold doses of the various vesicles ranged between 50 and 250 micrograms.

Table 3 summarizes the results of assays on collected fluid from Series IV. Culture No. 1 was the most active culture in the entire study. Within a seven-month interval, this culture showed an increase in gonadotrophic activity of 15,036 percent over the calculated activity of the explant. After more than 320 days *in vitro*, the culture was still secreting gonadotrophin.

Assays for estrogen proved negative.

TABLE 3.—*Summary of gonadotrophin assay results for Series IV*

Culture No.	Weight of tissue explant (*mg.*)	Threshold dose of homogenized tissue (*μg.*)	Calculated activity of explant (*M.U.U.*)	Total culture-fluid activity (*M.U.U.*)	Percent increase in activity
1	29.38	200	147	22,250	15,036
2	22.78	250	91	1,483	1,530
3	12.56	250	50	91	78
4	15.72	50	314	1,256	300

Cytological Observations

In some cultures, irregularly shaped, sheetlike cells with sharp spikelike processes were seen. These cells were agranular, with both cytoplasm and nuclei of such low refractive index as to be extremely difficult to see. An area of these cells from a culture that showed gonadotrophic activity is shown in figure 1. An area was photographed in one culture when it was

active and the same area (fig. 2) was again photographed six weeks later when the culture was no longer active. Comparison of the photographs showed no cell migration and a barely discernible rearrangement of the cell processes at both times.

A second category of cells seen were flat, often polygonal but sometimes elongate epithelial-like cells (figs. 3, 4, 13). These cells spread out on the glass interface and were irregular in shape and size. Many small granules of extremely uniform size were often grouped in a compact zone around the clear, rounded or ovoidal nucleus. Usually the cell edges could not be sharply defined, apparently because of contact with adjacent cells. Figure 3 shows an area of these cells from a culture whose fluids contained gonadotrophins; figure 4 shows an area of cells considered to be of the same type, but from a negative culture. The cells in figure 7 are also probably in the same category but are more or less rounded in shape. The cells in this category may greatly enlarge (fig. 13), become polynucleate and increasingly granular, and in some instances form large granular "giant cells."

Another category of relatively large cells (figs. 5 and 6) varied greatly in size as well as in shape. Sometimes they were sheetlike, at other times they were spherical. The cytoplasm was finely granular and often heavily vacuolated, with the vacuoles ranging from small to large. The densely granular zone appears as a ring surrounding a finely and lightly granular central area. These cells are so densely granular that it was often difficult to recognize the nucleus, but it appeared to be a small, oval body with a clearly defined nucleolus.

Figure 8 shows a group of large agranular cells with what appeared as a slightly fibrillar cytoplasm and a small oval nucleus and highly refractile nucleolus. This cell type was frequently seen but its appearance did not clearly suggest any relationship with any of the other cell categories seen.

Figures 9 and 10 show one type of tissue architecture frequently seen in both gonadotrophin-secreting and nonsecreting cultures; figures 11 and 12 show another type.

From these visual examinations and photographic records of the living unstained cells, several cell categories and patterns were observed. Each of these was seen irrespective of whether the culture did or did not have gonadotrophic activity; no cell or architectural type could be considered exclusively characteristic of cultures with hormonal activity.

Discussion

The quantitative data demonstrate that hydatid mole grown in an entirely heterologous culture medium produced gonadotrophic hormone over an extended period of time. Increases in gonadotrophic activity ranging from 600 to 15,000 percent showed that it could not be attributed to biological variations.

Since gonadotrophic hormone activity was present in cultures that had been incubated under sterile conditions, it is apparent that heat alone at 38° C. will not destroy the hormone under these conditions. Consequently,

inactivity of certain cultures in these studies should be attributed to lack of hormone production rather than to its destruction by either heat or bacterial infection.

Gey, Seegar, and Hellman (*8*) and Jones, Gey, and Gey (*9*) demonstrated the production of gonadotrophin from the hydatid mole after 37 days of culture, and possible gonadotrophin production after 62 days. In all instances the media used contained human serum. In the present study gonadotrophin was recovered from cultures more than one year after explantation, even though the cultures were growing in an entirely heterologous culture medium. The cells of the culture were able to utilize constituents of this heterologous medium to continue elaboration of the hormone.

Gonadotrophic activity of the mole could not be predicted from the potency of the original tissue homogenate since Series IV, in which the largest amounts of tissue were required for a threshold response, proved to have the greatest secretory activity in the culture fluids of any series in the study. Further, fluids from one group of cultures varied greatly in secretory activity from time to time. Mole tissue from the same patient obtained six months apart showed varied amounts of activity (tables 2 and 3). At the present time the factors responsible for the variations and fluctuations of activity have not been indicated by data obtained.

In most tissue culture studies, attention has been centered largely on the outgrowth from the explants. However, much work has been done on the study of explanted tissues themselves and it has been shown that such tissue will continue to live, differentiate, and function. In many such cases the growth stimulants were reduced to a minimum so that proliferation would be discouraged. Fell (*10*) has extensively reviewed the literature in this field. In the present study the amount of embryonic extract was maintained at levels that normally would promote growth for many cell types, but proliferation was minimal in the cultures of this study. Some cultures showed rapid migration of cells from the explant while others exhibited little or none. Even with this minimal proliferation the conditions were extremely satisfactory for the maintenance of secretory activity.

Several possibilities exist to explain inactive cultures or those showing a decline and subsequent loss of activity. Such cultures may contain so low a population of gonadotrophin-secreting cells that the amount of hormone secreted may be nondetectable by this sensitive assay method. Another possibility is that some explants were better able to adapt to *in vitro* conditions and to continue functioning as *in vivo*.

Summary and Conclusions

1) Gonadotrophin was recovered from one culture that had been *in vitro* for 413 days, and from two other cultures that had been *in vitro* for 377 days.

2) A sensitive method for measuring gonadotrophic hormone was adapted to tissue culture so that the activity of each culture could be

evaluated quantitatively in terms of the amount of tissue originally planted.

3) One culture showed an increase in gonadotrophic activity of 15,036 percent over that planted. Others showed increases of from 0 to 1,530 percent, including two cultures with increases of 1,093 percent and 983 percent.

4) Limited study of the types of cells present in unstained living cultures did not indicate any particular cell type as the source of the gonadotrophic hormone.

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PLATE 86

FIGURE 1.—Photograph of an area of a culture *in vitro* for 2 months, showing irregularly shaped, flat cells with spikelike processes. Fluid diluted 1:100 on bioassay showed gonadotrophic activity. × 200

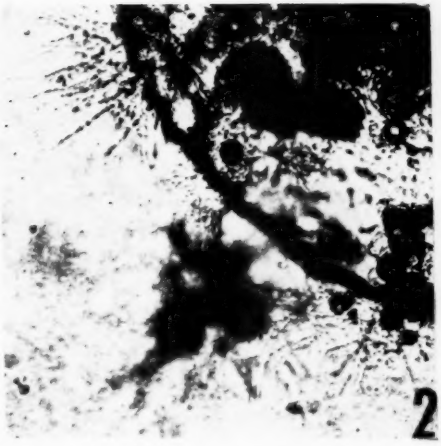
FIGURE 2.—Photograph of an area of a culture *in vitro* for 4 months. The flat cells in this culture also show spikelike processes. Fluid diluted 1:10 on bioassay showed no gonadotrophic activity. × 200

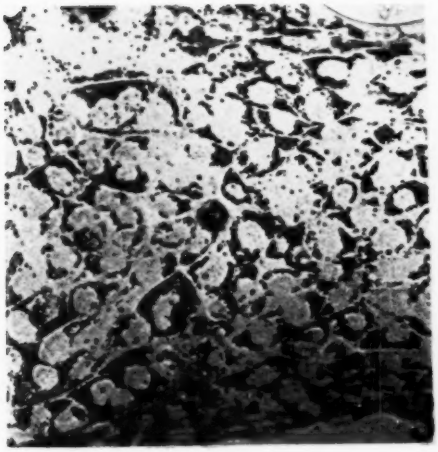
FIGURE 3.—Photograph of an area of a culture *in vitro* for 4 months. Note the small, extremely uniform granules compactly grouped around the nucleus of the epithelial-like cell. Fluid showed gonadotrophic activity. × 200

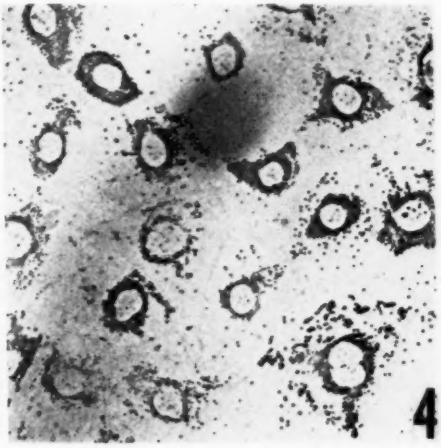
FIGURE 4.—Photograph of an area of a culture *in vitro* for 8 months. The cells in this culture appear similar to those in figure 3. No gonadotrophic activity was present in the fluid. × 200

FIGURE 5.—Photograph of an area of a culture *in vitro* for 65 days. Note the wide range in size of the vacuoles and the fine granules in the large, flat or rounded, epithelial-like cells. Fluid diluted 1:300 on bioassay showed gonadotrophic activity. × 200

FIGURE 6.—Photograph of an area of a culture *in vitro* for 65 days. The cells appeared to be like those in figure 5. Fluid diluted 1:5 on bioassay showed no gonadotrophic activity. × 200







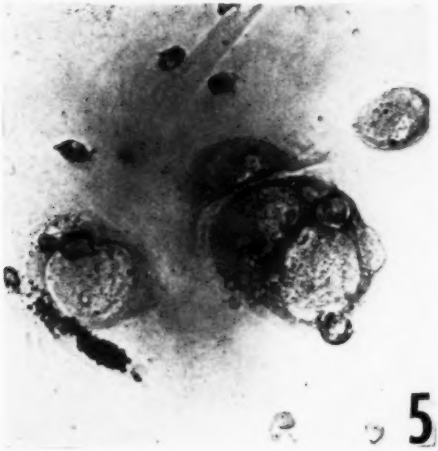


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PLATE 87

FIGURE 7.—Photograph of an area of a culture *in vitro* for 84 days. The cells are probably similar to those of figures 3 and 4 but are more rounded in shape. Fluid diluted 1:700 on bioassay showed gonadotrophic activity. × 200

FIGURE 8.—Photograph of an area of a culture *in vitro* for 36 days. This was ultimately the most active secretory culture in the series. Fluid diluted 1:300 on bioassay showed gonadotrophic activity. × 200

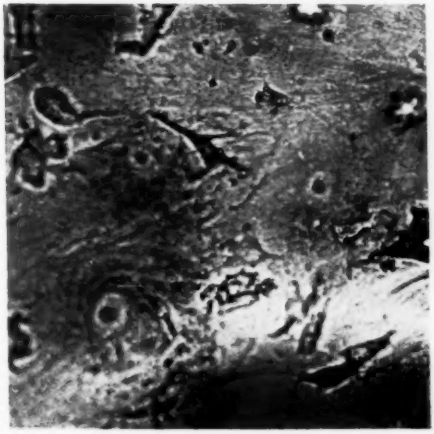
FIGURE 9.—Photograph of an area of a culture *in vitro* for 59 days. The tissue architecture of the cultures is typified by this figure. Fluid diluted 1:100 on bioassay showed gonadotrophic activity. × 200

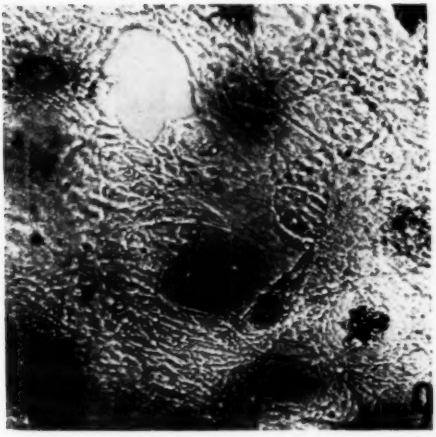
FIGURE 10.—Photograph of an area of the same culture as figure 9, after having been *in vitro* for 7 months. Fluid diluted 1:5 on bioassay showed no gonadotrophic activity. × 200

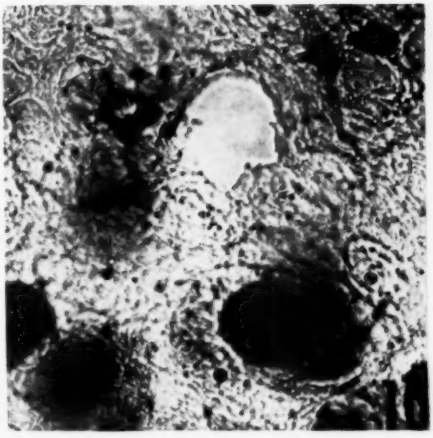
FIGURE 11.—Photograph of an area of a culture *in vitro* for 21 months. This culture typifies the tissue architecture of a culture with no secretory activity. × 200

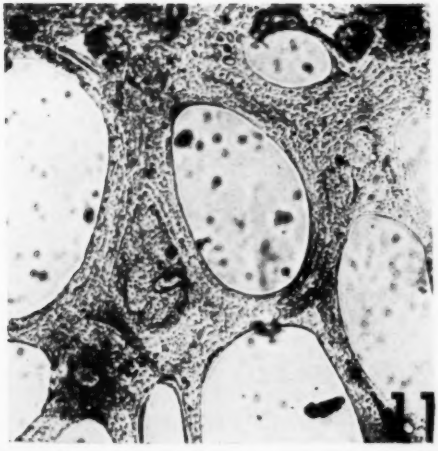
FIGURE 12.—Photograph of an area of a culture *in vitro* for 7 months. This culture typifies the tissue architecture of a culture with secretory activity. × 200

7









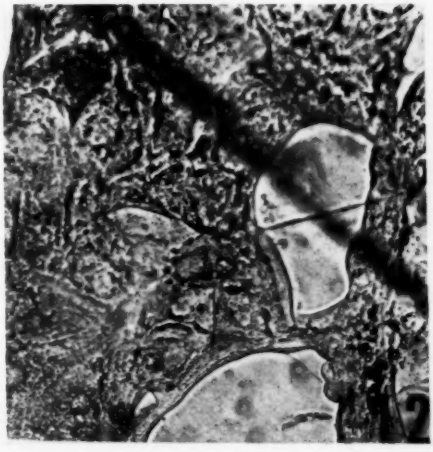


PLATE 88

FIGURE 13.—Photograph of an area of culture number 4 of Series I, after being *in vitro* for 4 months. Fluid collected from this culture 2 months later was positive for gonadotrophin when diluted 1:100. Note the great variation in the shapes and sizes of the cells and the large, polynucleate, granular cells. × 200

13

Leukemia Homotransplants in Mice[1,2]

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This communication reports the adaptation of spontaneous mouse leukemia of strain AKR (formerly designated strain RIL) to other mouse strains. The leukemia was adapted to adult (C × C3H)F_1 hybrid mice and to adult strain C3H mice by passage through newborn (C × C3H)F_1 hybrids. It was then adapted to adult strain A mice by passage through adult (A × C3H)F_1 and F_2 hybrid mice.

The greater susceptibility of young animals, as compared to the adults, to certain transplanted tumors has been demonstrated by others. In 1950 Rogers, Kidd, and Rous (*1*) showed that the carcinoma induced by the Shope papilloma virus could be adapted to resistant adult rabbits by one passage through mongrel suckling rabbits. That same year Gross (*2*) reported the susceptibility of suckling infant mice, and the resistance of adult mice of strain C3H to inoculation with AK leukemia. Shay *et al.* (*3*) made similar observations using infant rats.

Materials and Methods

The mice were all obtained from stocks maintained at the National Cancer Institute. The strain C3H mice were obtained from Dr. G. E. Jay, as were strain A subline Lilly which will be designated simply as strain A. The strain AKR mice were obtained from Dr. L. W. Law. The strain BALB/c mice, which will be designated simply as strain C, and the F_1 hybrids of C × C3H were obtained from Dr. Clifford Grobstein. Mice of both sexes from 5 to 9 weeks of age were used. Mice of this age group are designated as adult mice. All were kept under observation until death from the tumor growth or for at least 3 months. In some experiments they were kept for 12 months. The animals were housed in plastic cages and were constantly supplied with Purina laboratory chow and tap water.

The tumor used for the initial inoculation was an homogenate prepared by pooling the leukemic tissues—liver, spleen, thymus, mesenteric node,

[1] Received for publication October 8, 1953.

[2] This paper was presented at the meetings of the American Association for Cancer Research in April 1953, at Chicago, Illinois.

[3] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

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and peripheral lymph nodes—of 5 AKR mice bearing spontaneous leukemias and one bearing transplanted AKR leukemia No. 4616, which arose as a spontaneous AKR leukemia and had been carried through its eighth transplant. These leukemias were obtained from Dr. L. W. Law of the National Cancer Institute. The six leukemias were pooled because this preparation was also to be used for preparing a cell-free extract and filtrate for another project, in which it was attempted to produce leukemia in C3H mice with a cell-free preparation from AKR leukemias. The above tissues were homogenized in a Potter-Elvehjem homogenizer and enough Ringer's-lactate [4] solution was added to make a 20 percent suspension. The large particles were allowed to settle and were used to inoculate adult mice. The remaining fine cellular suspension was used for inoculation of newborn mice.

Adaptation of Tumor to Adult (C × C3H)F_1 Hybrid Mice by Passage Through Newborn Mice of the Same Hybrid

In order to check the specificity of the AKR leukemia, twelve 6-week-old (C × C3H)F_1 hybrid mice and twelve 6-week-old mice of strain C3H were inoculated intraperitoneally with 0.2 ml. of the sediment from the 20 percent homogenate prepared from the six pooled leukemias as described above. These mice were kept under observation for one year and none developed leukemia.

At the same time, a litter of four newborn (C × C3H)F_1 hybrid mice was inoculated intraperitoneally with 0.1 ml. of the supernatant from the above homogenate, using a tuberculin syringe and a 27-gauge needle. Two mice died within 6 days after inoculation with no gross evidence of leukemia. The third died on the 17th day, and necropsy showed an enlarged liver and spleen. The fourth mouse, which was ill, was sacrificed on the 18th day. The liver, spleen, and mesenteric nodes were enlarged; the thymus appeared uninvolved. These tissues were ground in an homogenizer just enough to allow a 1:2 suspension in Ringer's-lactate solution to pass through an 18-gauge needle, and 0.2 ml. of this suspension was inoculated intraperitoneally into adult mice as shown in table 1. The tumor that developed in the adult mice as a result of the inoculation is referred to as RIL6. All further transplants through the 7th generation were carried out by the above procedure. After the 7th transplant adult mice were inoculated subcutaneously by trocar or by syringe and needle after mincing the tumor with scissors or passing it through a tumor press.

The susceptibility of adult (C × C3H)F_1 hybrid mice and adult mice of strains AKR, C3H, and C to the RIL6 tumor was determined following the one transfer through (C × C3H)F_1 newborn mice. It was found that the tumor now grew not only in the AKR strain of its origin, but also in adult (C × C3H)F_1 hybrids (table 1).

From the first tumor passage through the seventh in adult (C × C3H)F_1 hybrid mice, the tumor showed no change in its ability to grow only in

[4] Eli Lilly Co.

TABLE 1.—*Susceptibility of adult mice to RIL6 tumor after one passage through newborn (C × C3H)F_1 hybrid mice*

Mice	Number inoculated	Number developing tumor	Remarks
(C × C3H)F_1 hybrids	4	4	All sacrificed after 12–23 days for tumor transfer.
Strain AKR	3	3	Died with leukemia in 18–19 days.
Strain C3H	3	0	Observed for only 3 months.
Strain C	2	0	Observed for only 3 months.

the (C × C3H)F_1 hybrids and in its AKR strain of origin. All the transplants were made from the hybrid mice using preparations made from the livers and spleens of mice carrying the tumor. Death from the tumor occurred in 21 to 30 days.

After the seventh transfer the tumor was passed from an AKR mouse back to a litter of six newborn (C × C3H)F_1 hybrid mice. Eleven days after inoculation three of the infant mice were living but were very ill. They were sacrificed and an homogenate was prepared from the pooled livers and spleens, which were not enlarged. This homogenate was passed through a second litter of six newborn (C × C3H)F_1 hybrids. Thirteen days later only three of the mice were living, but these had very swollen abdomens. They were sacrificed, and an homogenate prepared from the pooled, enlarged livers and spleens was inoculated subcutaneously into ten adult (C × C3H)F_1 hybrid mice. Within 21 days, four of these mice developed large tumors at the site of inoculation. On further transplantation from all four of these mice, the tumor now grew not only in the (C × C3H)F_1 hybrids and the AKR mice, but also in adult strain C3H mice. This is shown in table 2.

Although the C3H adult mice were now susceptible to the tumor which had been adapted to the adult (C × C3H)F_1 hybrid mice, strain C mice remained resistant to the tumor. Strain A mice that were inoculated were also found to be resistant to the tumor.

TABLE 2.—*Susceptibility of adult mice to the RIL6 tumor after the second series of passages in newborn C × C3H hybrids followed by one passage through the adult hybrids*

Mice	Number inoculated	Number dying from tumor	Days required to kill
(C × C3H)F_1 hybrids	45	40	21–30
Strain C3H	15	12	21–30
Strain C	18	0	.....
Strain AKR	4	4	13–27
Strain A	18	0	.....

Attempts to adapt this tumor to adult mice of strains C and A, by passage through newborn mice of these strains, were unsuccessful. However, newborn strain A mice were highly susceptible to tumor transplants, all dying in 13 to 18 days. White-blood-cell counts taken prior to death were frequently as high as 200,000 to 300,000, and microscopic sections of the tissues showed heavy infiltration of tumor cells in the liver, spleen, and kidneys.

Tumor RIL6 was carried through five passages in newborn strain A mice with no marked change in its ability to grow in adult strain A mice. Local tumors developed at the site of transfer in all the mice inoculated, but in all instances the tumors regressed within 3 weeks.

Newborn strain C mice were found to be more resistant to the RIL6 tumor. The tumor was carried through only two successive transfers and about half of the infant mice inoculated at each transfer developed leukemia. Transplants from these to adult C mice caused local tumor formation in one out of six inoculated. The tumor regressed in one week.

After tumor RIL6 had been carried for 12 months or 21 passages in C × C3H or strain C3H mice, it was checked for specificity by subcutaneous inoculation into adult mice of different strains. The tumor grew progressively in all the inoculated mice of strains AKR and C3H, and in (C × C3H)F_1 hybrids, killing in 10 to 30 days. It did not grow in mice of strains C or A. Microscopic examination of sections made from the liver, spleen, and lymph nodes of mice dying from the tumor showed a heavy infiltration of leukemic cells in all these tissues.

Adaptation of RIL6 Tumor to Strain A Mice by Passage Through Adult (A × C3H)F_1 and F_2 Hybrids

Fifteen 5-week-old (A × C3H)F_1 hybrid mice, derived by mating strain A females with strain C3H males, were inoculated subcutaneously with 0.2 ml. of 1:2 concentration of RIL6 tumor mash (the 17th transplant in C × C3H and C3H mice). All these mice developed large tumors and died with the usual manifestations of leukemia within 30 days, except for two mice that were sacrificed for transfer of the tumor. After the sixth passage in (A × C3H)F_1 hybrids the tumor was inoculated into 30 adult (A × C3H)F_2 hybrids. The tumor was found to develop in, and to kill, 26 of the 30 mice inoculated. Two of the mice

TABLE 3.—*Susceptibility of adult mice to RIL6 tumor after 6 passages through (A × C3H)F_1 hybrids, and 1 passage through F_2 hybrid mice*

Mice	Number inoculated	Number dying from tumor	Days required to kill
(A × C3H)F_1 hybrids	40	40	23–30
(A × C3H)F_2 hybrids	30	26	23–30
Strain A	28	22	23–30

bearing the tumor were sacrificed for transfer of the tumor and were considered as having died of the tumor. Adult strain A mice inoculated with tumors from the F_2 hybrids were now found to be susceptible to the RIL6 tumor (table 3). Large tumors developed, which spread throughout the subcutaneous tissues of the abdomen and back. In 21.4 percent of the animals these tumors regressed after 3 or 4 weeks; in the others they killed the mice. Microscopic sections showed infiltration of tumor cells into the liver and spleen.

Discussion

In the adaptation of tumor RIL6 herein described, the AKR leukemia became adapted to adult mice of strain C3H and strain A and to adults of the hybrids (C × C3H)F_1, (A × C3H)F_1 and F_2, but after 2 years' passage in (C × C3H)F_1 hybrids or C3H mice it still grew in and killed strain AKR mice, producing typical leukemia. This is summarized in Chart 1. Tumor No. 4616 (the transplanted AKR leukemia incorporated in the original homogenate) was carried for one year in AKR mice without any change in its specificity. The tumor RIL6 represents an homogenate prepared from six pooled AKR leukemias; the original homogenate failed to grow in either twelve 6-week-old strain C3H mice or twelve 6-week-old (C × C3H)F_1 hybrids when inoculated intraperitoneally. Whether the RIL6 tumor, or the tumor recovered after one passage through the newborn (C × C3H)F_1 hybrid mice, represents all six of the pooled AKR leukemias present in the original homogenate or only one or two of these cannot be ascertained. But, since the original homogenate failed to grow in adult strain C3H mice or adult (C × C3H)F_1 hybrids, it is probably of no consequence how many leukemias it represents.

Whether the tumor cells in the process of adaptation underwent some sort of physiological change that enabled them to grow in strains previously resistant to the tumor cannot be ascertained, but if a change occurred it was of such nature that it did not hinder the ability of the tumor cells to grow progressively in their strain of origin, *i.e.* AKR mice. The presence of a filterable agent that under certain conditions produces leukemias in the absence of tumor cells (*4*, *5*) may enhance the process of adaptation of the tumor cells. This enhancement may be due to the effect of toxicity or virulence of the agent on the host, thus enabling the tumor cells to grow in foreign strains.

Summary

1) A suspension of AKR leukemia cells prepared from 6 AKR tumors was adapted to adult (C × C3H)F_1 hybrid mice after one passage through newborn (C × C3H)F_1 hybrids.

2) Adult C3H mice were also found to be susceptible after two additional passages of the tumor through newborn (C × C3H)F_1 hybrid mice.

3) Attempts to adapt the tumor to adult mice of strains A and C by passage through newborn mice of these strains were unsuccessful. Newborn strain A mice were highly susceptible to the leukemia.

CHART 1.—*Summary on mouse passage of RIL6 tumor and its adaptation to adult mice of other strains or their hybrids*

Strain or hybrid in which grown	No. of transplants in given strain or hybrid	Strain or hybrid of adult mice susceptible
Newborn (C × C3H)F_1	1	AKR, (C × C3H)F_1
Adult (C × C3H)F_1	7	AKR, (C × C3H)F_1
Adult AKR	1	
Newborn (C × C3H)F_1	2	AKR, (C × C3H)F_1, C3H
Adult C3H or (C × C3H)F_1	17	AKR, (C × C3H)F_1, C3H (A × C3H)F_1
Adult (A × C3H)F_1	6	AKR, (C × C3H)F_1, C3H, (A × C3H)F_1 or F_2
Adult (A × C3H)F_2	1	AKR, (C × C3H)F_1, C3H, (A × C3H)F_1 or F_2, A
Adult A	1	
AKR		

4) It was found possible to adapt the tumor to adult strain A mice by passage through adult (A × C3H)F_1 and F_2 hybrids.

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An Agent from *E. coli* Causing Hemorrhage and Regression of an Experimental Mouse Tumor. V. Behavior of the Agent in the Presence of Various Reagents [1,2]

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The agent obtained from cultures of *Escherichia coli*, which is capable of producing hemorrhagic lesions in the tumors of mice implanted with mouse Sarcoma 180, is now known to be a complex polysaccharide containing both a peptide and a phospholipid moiety (*1–4*). It has also been shown that when this agent is treated with 25 percent aqueous trichloroacetic acid at 25° C. the peptide moiety is largely removed, leaving a lipopolysaccharide of approximately the same tumor-hemorrhagic activity as the original agent (*1*). This communication presents a further account of the action of various chemical and enzymatic reagents on the mouse tumor-hemorrhagic agent from *E. coli*, and from these studies it appears that hemorrhagic activity is retained only in those cases where the lipopolysaccharide is not destroyed.

When a solution of the hemorrhagic agent in 0.1 *M* aqueous hydrochloric acid is heated at 37° C. for 18 hours, a substantial proportion of the original hemorrhagic activity is retained and there is no indication of any significant amount of hydrolytic degradation (*cf.* table 1). However, comparable treatment of a solution of the agent in 0.1 *M* aqueous sodium carbonate (*p*H 10.9) results in a complete loss of tumor-hemorrhagic activity with definite evidence for the concomitant hydrolytic degradation of the lipopolysaccharide (table 1). From the experiment in which the agent was subjected to the action of pancreatic lipase at 37° C. and *p*H 8.9 for 18 hours, *vide post*, it may be inferred that the agent is as stable at *p*H 8.9, insofar as tumor-hemorrhagic activity is concerned, as it is at *p*H 1. Thus, the tumor-hemorrhagic agent from *E. coli* appears to be reasonably stable in aqueous solutions at 37° C. for an 18-hour period if the *p*H of the solution is in the region from *p*H 1 to *p*H 9, and is destroyed only if the *p*H of the solution is substantially greater than the latter value.

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[3] Contribution No. 1855.

TABLE 1.—*Action of various reagents on the hemorrhagic agent*

Treatment	P (*percent*)	Tyrosine (*equiv. percent*)	Glucose (*equiv. percent*)	Glucosamine (*equiv. percent*)	Chloroform (*soluble percent*)*	Activity (*γ/mouse*)
None†	1. 5–1. 7	1. 3–1. 5	52–55	15–17	0	0. 05–0. 025
HCl; 37° C.	1. 5	1. 35	56	12	0	0. 1
Na_2CO_3; 37° C.	1. 5	1. 3	62	13	1. 7	inact.
H_2O; (*p*H 7) 60° C.	1. 5	1. 3	58	12	0	1
H_2O; (*p*H 4) 100° C.						
Fraction II	0. 85	0. 5	83	15	.....	inact.
Fraction III	1. 6	5. 4	.....	.....	.....	
Fraction IV	0. 3	.....	.....	.....	13	
$ClCH_2CO_2H$; 100° C.	0. 5	0. 2	93‡	14	19	inact.
H_2O_2	1. 8	0. 9	56	17	.....	1
$HCONH_2$	1. 0	1. 6	63	17	1. 2	10
$NaIO_4$						
Fraction II	2. 0	.....	24	6	.....	
C_6H_5OH						
Fraction I	1. 65	0. 7	61	15	.....	100
Fraction II	1. 3	1. 3	.....	.....	.....	
Fraction III	1. 65	11. 8	.....	.....	.....	.....
Fraction IV	.....	.....	.....	.....	3. 5	
Fraction V	1. 8	1. 3	71	.....	.....	100
Trypsin	1. 7	0. 9	56	16	.....	0. 025
Chymotrypsin	1. 8	1. 2	53	14	.....	0. 025
Pancreatic extract	1. 7	1. 2	57	12	0	0. 1
Pancreatic lipase	1. 6	1. 3	56	17	0. 6	0. 1

* In reaction product before hydrolysis.

† *Cf.* reference (*1*).

‡ 8% equivalent glucose before hydrolysis.

The hemorrhagic agent was partially inactivated when it was heated at 60° C. for 3 hours in the presence of a phosphate buffer of *p*H 7.0 without evidence of hydrolytic degradation by the criteria employed in this study (*cf.* table 1). However, when an aqueous solution of the agent (*p*H 4.0) was heated at 100° for 1 hour, all hemorrhagic activity was lost with the obvious degradation of the agent as shown by the isolation of a carbohydrate-rich fraction (II), a protein-rich fraction (III), and a lipid fraction (IV) from the hydrolysate. When the agent was heated at 100° for 3 hours with 0.1 *M* aqueous monochloroacetic acid, all activity was lost and it was apparent (table 1) that degradation had proceeded to the point where the carbohydrate moiety was being hydrolyzed.

Treatment of the agent with dilute aqueous hydrogen peroxide caused a significant loss in tumor-hemorrhagic activity. However, the extent of inactivation was somewhat less than that observed when the agent was subjected to prolonged treatment with anhydrous formamide. Although the activity of the agent was diminished and a small amount of a lipid fraction was liberated by the latter treatment, it should be noted that, in common with the hemorrhagic agent obtained from *Serratia marcescens* (*5*), the lipid moiety of the hemorrhagic agent isolated from *E. coli* is much more firmly bonded to the remainder of the agent than is the lipid moiety of the antigenic complex of the Shiga bacillus which Morgan and Partridge (*6*) were able to separate by treatment of the complex with anhydrous formamide.

The hemorrhagic agent from *E. coli* is rapidly attacked by periodate, but the reaction product is so insoluble in water, or in dilute aqueous sodium hydroxide, that it was impossible to prepare a solution suitable for the determination of hemorrhagic activity. It may be noted that a similar situation was encountered in the oxidation of blood group A substance preparations with periodate (*7*).

Treatment of the hemorrhagic agent at 25° C. with 90 percent aqueous phenol leads to a substantial loss in hemorrhagic activity and a partial cleavage of the agent into its component parts. While the greater part of the agent was insoluble in the reagent, there was obtained from the phenol-soluble fraction a protein-rich fraction and a lipid fraction (table 1).

Hartwell, Shear, Adams, and Perrault (*5*) found that the mouse tumor-hemorrhagic agent isolated from *S. marcescens* was stable in the presence of crude pancreatic trypsin, pepsin, and papain, insofar as tumor-hemorrhagic activity was concerned, and these authors employed a digestion with crude pancreatic trypsin in the purification of the agent. An attempted digestion of the hemorrhagic agent from *E. coli* with crude pancreatic trypsin, or with crystalline α-chymotrypsin, led to products that were as active as the original preparation. While the above treatments were without detectable effect upon the tumor-hemorrhagic activity, it must not be inferred that the agent was not attacked by these enzymes, but rather that the attack may have been limited to the peptide moiety which is not essential for tumor-hemorrhagic activity (*1*).

Treatment of the hemorrhagic agent with a crude pancreatic extract appeared to cause a slight decrease in hemorrhagic activity as did treatment with a preparation of crude pancreatic lipase, which in addition led to the liberation of a small amount of a lipid fraction. However, in either case there was no evidence for extensive degradation and the slight loss in activity could easily have been due to factors other than the action of the enzyme, *i.e.* the temperature and the *p*H of the reaction mixture.

The retention of hemorrhagic activity in the presence of the common pancreatic enzymes is not surprising when it is remembered that in one step in the isolation procedure (*1*), wherein the culture is concentrated with the bacterial cells still present, there is considerable autolysis which appears to be without effect upon the hemorrhagic activity of the agent.

The results of the present investigation are consistent with the proposition that hemorrhagic activity is a property of the lipopolysaccharide that can be obtained from the original hemorrhagic agent by treatment with 25 percent aqueous trichloroacetic acid at 25° C., and that any treatment resulting in the destruction of the lipopolysaccharide, either wholly or in part, also causes a loss or decrease in hemorrhagic activity. These findings are similar to those of Zahl (*8*) who showed that the tumor-hemorrhagic activity of the somatic antigenic complex of Type Z *Shigella paradysenteriae* decreased with increasing degradation of the complex, and that a preparation which had most of the protein and approximately 40 percent of the bound phosphorus removed possessed approximately one-

half the activity of the original complex. Tal and Olitski (*9*) also found that in the case of the antigenic complex from *Shigella dysenteriae* the closely allied Shwartzman phenomenon was exhibited by fractions containing phosphorus bound to carbohydrate, but not by fractions containing phosphorus bound to protein. However, in the production of adrenal and placental hemorrhagic reactions, activity was associated with the fraction containing phosphorus bound to protein.

Experimental

Starting materials and general techniques.—The mouse tumor-hemorrhagic agent employed in these studies, unless otherwise noted, was the ethanol-fractionated material previously designated as fraction B_1 (*1*). In the experiment with trypsin a preparation that had not been subjected to ethanol fractionation, *i. e.* fraction A_1 (*1*), was used.

The methods employed for the determination of percent phosphorus, equivalent percent tyrosine, equivalent percent glucose (reducing sugar), and equivalent percent glucosamine were those described previously (*1*).

In general the reaction products were isolated by a procedure based on thorough dialysis of the reaction mixture against distilled water, the addition of sufficient dilute hydrochloric acid until the solution was acid to congo red, or alternately, the addition of 1 percent by volume of 50 percent (w/w) of aqueous trichloroacetic acid, and precipitation of the product by the slow addition, with stirring, of 1.5 volumes of 95 percent ethanol. The precipitates were collected by centrifugation, washed successively with ethanol and with acetone, and dried *in vacuo* over phosphorus pentoxide. In those cases where the amount of chloroform-soluble material liberated during the course of the reaction was determined, the chloroform extraction was conducted after the acidification of the dialyzed reaction mixture with dilute hydrochloric acid and prior to the precipitation of the product with ethanol.

The conditions used in the various reactions are given below.

Hydrochloric acid at 37°.—A solution of 99 mg. of fraction B_1 in 20 ml. of 0.1 *M* hydrochloric acid was incubated at 37° C. for 18 hours.

Aqueous sodium carbonate at 37°.—A solution of 75 mg. of fraction B_1 in 20 ml. of 0.1 *M* aqueous sodium carbonate (*p*H 10.9) was incubated at 37° C. for 18 hours.

Water at 60°.—A solution of 75 mg. of fraction B_1 in 15 ml. of a phosphate buffer of *p*H 7.0 (μ=0.1) was heated at 60° C. for 3 hours.

Water at 100°.—A solution of 500 mg. of fraction B_1 in 50 ml. of water (*p*H=4.0) was heated on a boiling water bath for 1 hour; after heating for 20 minutes a flocculent precipitate began to appear. The reaction mixture was cooled, the precipitate collected and dried to give 148 mg. (29 percent) of fraction I. The supernatant liquid was dialyzed against distilled water and lyophilized to give 266 mg. (53 percent) of fraction II. The precipitate, *i.e.* fraction I, was extracted with chloroform to give 80 mg. (16 percent) of an insoluble residue (fraction III) and a chloroform extract that contained 65 mg. (13 percent) of solids. The residue obtained

by removal of the solvent from the chloroform extract was extracted with acetone to give 53 mg. (10.5 percent) of an insoluble residue (fraction IV), which was a pale yellow powder.

Monochloroacetic acid at 100°.—A solution of 90 mg. of fraction B_1 in 25 ml. of 0.1 *M* aqueous monochloroacetic acid (*p*H 2.0) was heated at 100° C. for 3 hours.

Hydrogen peroxide at 37°.—To a solution of 100 mg. of fraction B_1 in 10 ml. of water was added 10 ml. of 2 percent aqueous hydrogen peroxide and the reaction mixture was incubated at 37° C. for 18 hours.

Anhydrous formamide at 5°.—A solution of 128 mg. of fraction B_1 in 5 ml. of anhydrous formamide was allowed to stand at 5° C. for 16 hours, 5 ml. of chloroform was added, the mixture was shaken and then centrifuged. To the upper formamide layer was added an equal volume of 95 percent ethanol; the flocculent precipitate was collected, washed successively with ethanol and with acetone, and dried to give 103 mg. (80.5 percent) of product. To the clear lower chloroform phase was added an additional volume of chloroform; the small amount of gelatinous precipitate that remained was discarded, the clear solution evaporated to dryness, and the residue taken up in chloroform; the solution was filtered and again evaporated to dryness to give 1.5 mg. (1.2 percent) of residue. The main fraction recovered from the formamide treatment was observed to be more readily soluble in water, and to give a clearer solution, than fraction B_1.

Aqueous sodium periodate at 25°.—To a solution of 1 gm. of fraction B_1 in about 30 ml. of water was added 15 ml. of 0.33 *M* periodate. The solution was made up to 50 ml. and placed in a 25° C. bath. When the titration (*8*) of 2-ml. aliquots at various time intervals indicated that the rapid phase of the reaction had ceased (after 2–3 hours), all subsequent aliquots were preserved, after titration, in order that they could be combined with the remainder of the solution when it had been titrated after a 24-hour time lapse. In this way 88 percent of the volume of the original solution was preserved. The total consumption of periodate after 24 hours was 4.1 *mM* per gram of fraction B_1. The stable foam that had formed during the titration was collected, washed with acetone, water, again with acetone, and dried to give 23 mg. (2.6 percent) of a light-brown powder (fraction I). The solution after the removal of the foam was thoroughly dialyzed against running tap water, and then against distilled water. The addition of 1 percent by volume of 50 percent (w/w) aqueous trichloroacetic acid to the dialyzed solution gave a flocculent precipitate which was collected, washed successively with ethanol and with acetone, and dried to give 286 mg. (32.5 percent) of a light-brown resin (fraction II) which was extremely insoluble in water and in dilute aqueous alkali.

Phenol at 25° C.—Twenty ml. of 90 percent (w/w) aqueous phenol was added to 400 mg. of fraction B_1; the mixture was triturated to effect dispersion and then centrifuged. The gelatinous pellet that had formed was first washed with 90 percent phenol, then with ethanol, then with

acetone, and finally dried to give 240 mg. (60 percent) of fraction I. To the combined phenolic extracts (30 ml.) was added 95 percent ethanol, slowly and with stirring, until 12 ml. had been added; the further addition of ethanol caused no further precipitation. The precipitate that had formed was collected, washed successively with ethanol and with acetone, and dried to give 23 mg. (6 percent) of fraction II. The supernatant liquid was dialyzed against distilled water, and the reddish precipitate that formed was collected, washed with water, dried, and extracted with chloroform to give 34 mg. (8.5 percent) of a reddish chloroform-insoluble residue (fraction III) and 14 mg. (3.5 percent) of a chloroform-soluble wax (fraction IV). The dialyzed solution remaining after the collection of the precipitate was lyophilized to give 38 mg. (9.5 percent) of fraction V.

Trypsin at 37°.—To 790 mg. of fraction A_1 in 36 ml. of water was added 10 ml. of 1 *M* disodium phosphate and 16 mg. of trypsin (1:300 powder, Nutritional Biochemicals Corp.). The mixture (*p*H 7.9) was incubated for 3 days at 37° C.

Chymotrypsin at 25°.—Sufficient 0.1 *M* aqueous sodium hydroxide was added to a solution of 96 mg. of fraction B_1 in 5 ml. of water to bring the *p*H of the solution to *p*H 8.0. One ml. of a 0.2 *M* tris-(hydroxymethyl)-amino-methane-hydrochloric-acid buffer of *p*H 8.0 (*11*) and 5.3 mg. of crystalline α-chymotrypsin were then added, the volume made up to 10 ml. with water, and the mixture allowed to stand at 25° C. for 4 days.

Pancreatic extract at 37°.—To 1 gm. of pancreas powder (dehydrated and desiccated 40° product, Viobin Corp.) was added 10 ml. of a 0.1 *M* disodium phosphate–monosodium phosphate buffer of *p*H 7.4, and the insoluble material removed by centrifugation. A mixture of 1.5 ml. of the clear extract, 75 mg. of fraction B_1, and 15 ml. of the phosphate buffer of *p*H 7.4 was incubated at 37° C. for 18 hours.

Pancreatic lipase at 37°.—To 101 mg. of fraction B_1 in 20 ml. of a 0.1 *M* ammonium chloride–ammonium hydroxide buffer of *p*H 8.9 was added 51 mg. of lipase powder (Nutritional Biochemicals Corp.), and the mixture was incubated at 37° C. for 18 hours. The reaction product was isolated from the digestion mixture after first removing the precipitate by centrifugation. The lipase preparation hydrolyzed 0.01 milliequivalents of methyl butyrate per ml. of enzyme preparation in 20 minutes at *p*H 8.9.

Summary

From a study of the action of various chemical and enzymatic reagents upon the mouse tumor-hemorrhagic agent from *E. coli*, it has been concluded that tumor-hemorrhagic activity is associated with the presence of an intact lipopolysaccharide.

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Deceased

February 12, 1954

The February 1955 issue of the *Journal of the National Cancer Institute* is being planned as a memorial issue for DR. LORENZ, and will contain original contributions from his colleagues presenting research done in cooperation with Dr. Lorenz or with which he had some association. The Editors also plan to include other papers in the field of radiobiology that would be appropriate for this commemorative issue.

Papers on original work concerning any of the many aspects of radiobiology with which Dr. Lorenz was associated will be eligible. Manuscripts to be considered for this memorial issue should be submitted no later than October 15, 1954.

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